INDUSTRIAL MICROBIOLOGY

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By

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SECOND EDITION

INDUSTRIAL MICROBIOLOGY

PREFACE TO THE SECOND EDITION

Since the publication of the first edition of this work in 1940, marked advances have been made in the subject by numerous investigators in many countries. The increase in interest in this field of microbiology which has thus been demonstrated and the wide range of the researches which have been carried on supply abundant proof of the recognition of the potential importance of fermentations and related processes in industrial biochemical operations. In this edition we have tried to keep pace with this gratifying extension of knowledge and to review it now in a form which may be useful to students in this branch of technology and to those concerned with research or technical application therein.

The inclusion of all this new material has made it desirable to revise and consolidate much of the earlier material and to present in the tables relating to production statistics, fermentation products, etc., the latest available data. The chapters on yeast have been rewritten into a single chapter and expanded by much new material on food and fodder yeasts, riboflavin production, and discussion of methods of operation industrially

Five new chapters have been added, giving extended descriptions of new processes of saccharification, recent work on yeast production and yeast products, the production and properties of 2,3-butanediol, the itaconic and related fermentations, and comprehensive discussion of antibiotics. All these have been prepared with special reference to industrial applications and the possibilities of future developments.

The revision of a book in a field showing such rapid development has of necessity increased the volume of material greatly, in this case about 50 per cent. There were 60 illustrations in the first edition, and the present volume contains 124

Grateful acknowledgment is made to the many authors and publishers who have kindly permitted use of quoted material, photographs, cuts, and tables.

We extend our thanks to the editors and publishers of scientific journals from which reproductions have been made, particularly to Dr. Walter J. Murphy, Editor of *Industrial and Engineering Chemistry;* the Williams and Wilkins Company, publishers of the *Journal of Bacteriology;* Dr. R. A. Bottomley, Managing Editor, *Cereal Chemistry;* Dr. Philip P. Gray, Wallerstein Laboratories; Dr. F. E. Nelson, Editor of the *Journal of Dairy Science;* and others. We are happy to acknowl-

PREFACE TO THE FIRST EDITION

For hundreds or even thousands of years mankind has practiced domestic arts in which microbes are the invisible but active agencies of desired change. Wine making, vinegar production, brewing, and the making of leavened bread were processes known to ancient peoples. Even when some of these processes became established on a larger scale the success was still somewhat dependent on chance and the combination of fortuitous circumstances with a degree of skill born of experience. Not until less than a hundred years ago was there any scientific appreciation of the real part microbes play in the transformations of organic matter.

Industrial microbiology is one of the important outgrowths of those fundamental researches conducted by Pasteur which have made his name the most highly revered in the whole realm of microbiology. Although his preeminence has been especially recognized in another field of microbic investigation which has greatly promoted man's welfare, the bacteriology of infectious disease, it should not be forgotten that his first studies were in fermentation and were conducted in aid of industries. He may thus especially be regarded as the founder of industrial microbiology as well as of medical bacteriology and immunology. Although the development of industrial microbiology was slow during the quarter century following Pasteur's work, a few outstanding contributions, such as those of E. C. Hansen, Jorgensen, Lafar, Delbrück, Duclaux, Lindner, and others, added materially to the subject before the opening of the present century. Since that time, development has been much more rapid, and industrial microbiology has now become a large and widely recognized field of study and practical application. In 1896 the senior author organized in the Biological Department at the Massachusetts Institute of Technology the first course of classroom instruction in industrial biology given in America. Of relatively small scope at the beginning, the course has been expanded from time to time as the subject has grown in importance, and the present related and parallel courses in industrial microbiology and food technology are outgrowths of its development.

The authors have prepared the present volume, believing that a comprehensive work in English dealing with this subject and presenting both theoretical and practical aspects of fermentation would be useful. Whether used primarily as a text for fairly advanced students or as a work

PREFACE TO THE FIRST EDITION

of reference, it is assumed in advance that the student or reader will already have had somewhat extensive training in biology and general bacteriology, and at least the fundamentals of organic chemistry and biochemistry. For this reason the general description and taxonomic treatment of the great class of the bacteria have been limited to the groups intimately concerned with the processes to be discussed later.

It is the purpose of this volume to outline, in a concise but comprehensive manner for students thus prepared, the fundamentals of industrial microbiology, and to present descriptions of the more important processes within the field.

No single text of moderate size can give a complete and detailed treatment of every aspect of the subject. There are in this volume what may, in some quarters, be regarded as serious omissions. For example, discussion of the commercial manufacture of sera, vaccines, and other therapeutic agents is omitted, as is also reference to the production of cultures of nitrogen-fixing bacteria, and of butter- and cream-ripening organisms, etc., although in a limited way each of these might be regarded as falling within the scope of industrial bacteriology. The reasons for these omissions will, it is believed, be evident on further thought—the first subject is closely related to public health and medicine; and the second subject seems more properly to belong to agricultural or to dairy bacteriology.

The efforts of the authors have been especially directed (1) to a broad treatment of those large-scale fermentations in which the end products are of themselves industrially or potentially important, and (2) to consideration of others that are of special significance because of the modifications of the quality of substrate materials that they produce, as in some aspects of food technology.

The various alcoholic fermentations as applied in brewing, wine-making, and distillation industries; the acetic fermentation; the lactic fermentation; and the fermentations yielding butyl alcohol, acetone, citric acid, glycerol, gluconic acid, etc., are described and discussed since they represent type processes that illustrate basic principles and may supply the key to modes of inquiry that may be useful to the student in later researches or in the pursuit of new aspects of industrial fermentations.

A more comprehensive treatment of the general aspects of the subject will be found in the introductory chapter.

Consideration is also given to the discussion of some of the more recently discovered fermentations which have been investigated and which seem to offer possibilities of industrial significance in the near future. These are treated not only from the standpoint of the organisms

involved but also with reference to the raw materials and production methods that may be employed and to the biochemistry of the reactions catalyzed by the microbes.

The work has attempted to present the subject in a unified way, although it divides somewhat naturally into four parts, the first three dealing, respectively, with the biochemical activities of yeasts, of bacteria, and of molds. Within each of these three classes of microorganisms with their extremely numerous and varied groups and species are found relatively few types having marked activity as causative agents of fermentation, but these are important since they represent types which probably can be employed economically and in large-scale operations. The last part of the book deals with specialized activities of microorganisms and some higher types of organisms in relation to undesirable changes produced in textile fibers, in wood itself, and in structures built of wood. Two appendices treat briefly of subjects useful to the industrial microbiologist. The first deals with the control of microbes by the use of germicides, and the principles of testing disinfectants, and the second with the treatment and disposal of industrial microbiological wastes.

At the end of each chapter, references are given, in order that the student who desires may continue his study of the subject by consulting original papers. The subject has now become so voluminous that it is impossible to present complete bibliographies, and although the references presented are regarded as the most useful for the student, it may be that numerous important publications have not been mentioned here.

Dr. A. Guilliermond, Dr. Ö. Winge, Dr. O. Meyerhof, Dr. H. Raistrick, Dr. C. G. Anderson, and Dr. Marjory Stephenson, for their friendly cooperation in supplying photographs, tabular material, and other data. Finally, the authors acknowledge with thanks the cooperation of their departmental colleagues, of Mrs. Cecil G. Dunn, who has made many suggestions and given unlimited cooperation in reading the proofs, and of Miss Janet Hartwell, who has rendered exceptional service in the preparation of the manuscript.

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CHAPTER I

INTRODUCTION

Industrial microbiology is that portion of microbiological science which deals with the possible utilization of microorganisms in industrial processes, or in processes in which their activities may become of industrial or technical significance. Obviously the term "microbiology" in its broadest sense comprises that division of biological science which treats of the extremely small organisms of both plant and animal natures that Sedillot in 1878 grouped together under the inclusive term "microbes." Although this word is now often used synonymously with bacteria it is not so limited, since within its field may actually be included all types of very small living things, such as the protozoa, spirochetes, and minute parasitic worms, as well as those low types of fungi commonly spoken of as the "yeasts" and "yeast-like organisms," the somewhat indefinite group called "molds," and the extremely numerous and wide-spread group of the bacteria. The modern general use of the term "protozoology," which has resulted from the many careful studies of the lowest group of animal organisms, has to a great extent in recent years transferred the interest and extended descriptions of many of the types of the animal group of *Protozoa* from the general field of microbiology to this more specialized domain of study. A modern tendency apparently is to confine the term "microbiology" largely to organisms that fall within the realm of the lowest groups of botanical or plant life. It is in this sense that the word is used in this book.

For several decades it has been known that numerous kinds of yeasts, molds and other low fungi, and several types or groups of bacteria have direct relation, either favorable or unfavorable, to certain types of economic processes carried out in connection with industrial or factory operations such as brewing, wine making, cheese making, etc., which have grown up from small-scale or family arts. Knowledge in this field has greatly extended in recent years, as research in many countries and in many lines has been carried out. The scale of operations has greatly enlarged and concentrated in manufacturing plants Industries have replaced household arts. These changes have made it evident that industrial microbiology is not only an exceedingly interesting branch of study but that it has already become a distinctly important branch of applied science, and one with even **greater** potentialities

In any broad-gauge discussion of industrial microbiology it should be emphasized at the outset that knowledge in this field is of usefulness and significance from two diametrically opposite angles of application. On the first and more positive side, it is clearly advantageous to know the biological and biochemical characteristics of the many types of organisms that are the prime and direct causes of chemical transformation of materials into desired products. Here one utilizes the microorganisms capable of producing, by fermentation processes, relatively large quantities of chemical substances of usefulness and economic value. In this type of process the biochemical methods are in some instances the only economic methods of production. The manufacture of industrial alcohol and other industries based on alcoholic fermentation may be taken as a type of this class. Of equal interest to the microbiologist is the part played by organisms of these groups in composite operations, where the action sought is the production of somewhat smaller quantities of desirable by-products that should occur only as minor but important components in the final product. This is the aim in some branches of food manufacturing and in other commodity products.

On the other hand, it is recognized that fermentations incited by microbes may not always be desirable but may be quite the reverse. Competition by invading contaminants may wreck what would otherwise be a successful process. It is, then, of much importance to have knowledge of the organisms specifically useful in a process, and to be equally informed regarding those which oftentimes are destructive to or detrimental in manufacturing processes and are the causes of economic loss. The trained industrial microbiologist must be able to discover them, to recognize the type of damage they produce, and to become versed in the methods of combating them.

With all these viewpoints in mind, it is clear that industrial microbiology should include in its scope the study (I) of the numerous fermentation processes in which the production of alcohols, organic acids, glycerol, acetone, and other substances are end products; (II) of certain aspects of food-manufacturing processes, such as baking and the making of cheeses, butter, sauerkraut, and pickles, in which microbic agencies take a significant and important accessory part; (III) of food-conservation methods, such as canning and preserving, refrigeration, quick freezing, and drying, where sterilization or inhibition is imperative; and (IV) of the microbiological problems concerned with textile and commercial fibers. Obviously in the processes grouped under (II) and (III) and in a part of those under (I) industrial microbiology is intimately associated with food technology. The work of the industrial microbiologist may also deal with the production and technical uses of bacterial and fungus enzymes,

such as amylases and proteases, or with the preservation or protection of wood and the processes of commercial disinfection, wherein the application of suitable chemicals to restrain or prevent fungus, bacterial, or enzyme activity on walls and floors or on materials undergoing processing in the mill or manufacturing plant may make the difference between success and failure of operation.

The production of sera, vaccines, and other therapeutic agents commercially, although generally *regarded as in the field of public health or medicine*, and hence not here treated, might in reality be considered as a group of processes in industrial biology. It has seemed best to omit these processes in this volume, and also not to include the study of soil microbiology, the use of nitrogen-fixing bacteria, and the study of the phytopathology of economic crop plants, use of disinfectant or inhibitive sprays, etc. Although all these aspects of microbiology are extremely important in agriculture and therefore constitute a part of economic microbiology, they do not fall within the limits of this work.

In the present volume, industrial microbiology will be regarded essentially as the science and art of investigating and controlling technical fermentations, that is, of using microorganisms as reagents to produce desirable end products having possible or well-defined industrial uses and applications. Obviously, conditions in which losses due to contaminations or wholesale microbic infection affect manufacturing operations must enter into the subject matter. Although all the minute details of particular industries cannot be dealt with in a text of this scope, an attempt is made to present the principles and the general methods of operation. In addition to the consideration of the organisms and biochemical reactions concerned in the better-known industrial fermentations now in use, a few fermentations of present theoretical interest but perhaps potentially capable of technical development are also discussed.

It is important not to make too broad assumptions regarding industrial uses of microbes and to keep in mind the special qualifications which must be possessed by organisms in order that they may be economically utilized in the direct industrial production of materials having distinct commercial value. Obviously the number of species having this valuable property must be limited. The industrially important microbes may be characterized as having at least three outstanding qualities:

1. The ability to grow rapidly in suitable organic substrates and to be easily cultivated in large quantity

2. The ability to maintain physiological constancy under these conditions and to produce the necessary enzymes readily and profusely, in order to bring about the desired chemical changes.

3. The ability to carry out these transformations under comparatively

simple and workable modifications of environmental conditions; and, since the reactions are exothermic, without the application of large quantities of external energy.

Obviously, industrial operations with microbes are more complicated than laboratory experiments but involve the same principles. Large-scale operations must include protection from contamination, and the chemical engineering equipments must be carefully designed to meet the special conditions required in each type of fermentation.

Hundreds of microbes can grow rapidly in solutions of organic substances without yielding significant quantities of valuable products. They may bring about decompositions that will be set up, but they may not be productive of a technically important or industrial fermentation.

Microorganisms of Fermentation.—As has been intimated, the microorganisms of fermentation include yeasts, molds, and bacteria. These microorganisms are unable to manufacture their own food by the ordinary process of photosynthesis since they lack chlorophyll and are classified as fungi, belonging to the phylum *Thallophyta*. Certain of the *Ascomycetes* (sac-fungi) and of the *Phycomycetes* (alga-like fungi), and a large number of species of the bacteria are the principal microorganisms that are directly concerned. A few of the *Basidiomycetes* are of significance in special types of breakdown of wood and fibers and are thus productive of changes comparable to fermentations.

The microorganisms of fermentation differ widely in respect to morphology, size, reaction to free oxygen, manners of reproduction, growth requirements, ability to assimilate or ferment raw (natural) substances, and in other ways. But they are similar in that they are "colorless" and grow most actively in darkness or diffused light, and all produce enzymes by which they catalyze the reactions ascribed to them.

Variation in Strains.—Even in a so-called "species" there may be a large number of types or strains, and, even in a so-called "pure-strain," variation may occur under different environmental and nutritional conditions. Although the usual mode of increase by asexual reproduction favors the constancy of a given species, many factors may tend to cause changes in the chemical composition of a microorganism, the type and quantity of end products formed, and the rate of growth and reproduction. For example, the temperature of incubation, the reaction of the medium, the oxygen relationships, the presence or absence of stimulating or inhibiting substances in the medium, and various other factors must be controlled and made similar in order to obtain analogous results with the same or related strains of a microorganism. The importance of mere traces of a sub-

stance cannot be too much stressed in some instances. Some of the apparent differences in results obtained in research in different laboratories with the supposedly same strain of organism may disappear when conditions of culture and the chemical composition of the media become exactly or essentially the same. It is, however, probably impossible to maintain perfect lack of variation over long periods.

Although some differences are only apparent ones, frequently true biologic variations exist in strains Too much work has been carried out by responsible laboratories to leave any doubt as to this fact. For example, some strains of a microorganism apparently require added growth-accessory substances, while other strains require none; some strains of *Aspergillus niger* are stimulated by iron and zinc salts, others receive no apparent stimulation from these salts.

Fermentation.—From the biochemical standpoint, fermentation is the name given to the general class of chemical changes or decompositions produced in organic substrates through the activity of living microorganisms Thus there may be many kinds of fermentation falling within this category depending on the type of organism involved, the type of substrate, or even the conditions imposed, such as pH, or oxygen supply. The word "fermentation" is a term that has undergone numerous changes in meaning during the past hundred years. According to the derivation of the term, it signifies merely a gentle bubbling or "boiling" condition, and the term was first applied when the only known reaction of this kind was in the production of wine. Even then no knowledge existed as to the cause. Thus in an active ethyl alcohol fermentation, as in a wine or cider fermentation, carbon dioxide is always liberated in the form of bubbles of gas, which at the height of the reaction may cause a marked agitation or movement of the liquid medium, especially in a large vat or tank, sufficient to give to it the appearance of a boiling liquid This interpretation of the word was the accepted one for several thousand years. After Gay-Lussac studied the process the meaning was changed to signify the breakdown of sugar into alcohol and carbon dioxide. With the increase in knowledge following Pasteur's researches as to the *cause* of this change in the nature of the material fermenting, the word became associated with microorganisms and still later with enzymes, which are the biologically produced reagents by which microbes work. For a long time fermentation was especially associated with carbohydrates and, indeed, is often so considered at present, but a broader conception of these biologic reactions seems to be more logical Thus putrefaction and the breakdown of fats by microbes are to be regarded as special kinds of fermentations

Although fermentation is frequently or even generally associated with

the evolution of gas due to the action of living cells, neither gas evolution nor the visible presence of living cells is today regarded as an essential criterion of fermentation. In certain fermentations, for example, some of the lactic fermentations, no gas is liberated. Again, fermentation might result (although it is not frequently the case) from the use of cell-free enzyme extracts that for a time catalyze the reactions. Gas can be liberated in some of these processes, as for example with a zymase preparation. Cell-free fermentations are, however, unknown in nature and fermentations where the living cells are present, and are largely of theoretical interest.

Fermentations are so varied in character that any broad definition that will cover this whole class of biochemical processes must be couched in very general terms. Nevertheless, it is sometimes useful to gain a briefly stated concept of such a group of reactions The idea might be expressed as follows:

A fermentation, in the broad sense in which the term is now used, may be defined as a process in which chemical changes are brought about in an organic substrate, whether carbohydrate or protein or fat or some other type of organic material, through the action of the biochemical catalysts known as "enzymes," elaborated by specific types of living microorganisms.

For more exact characterization, the kind of fermentation, such as alcoholic, lactic, acetic, etc., must be specified. Since the majority of fermentation processes first studied concerned carbohydrates, these are often regarded as the essential materials, but it must be clear that proteins, fats, some salts of organic acids, and alcohols can be broken down by similar agencies and thus fall within the classification of fermentable material.

Enzymes.—The student of biology or biochemistry will already have learned of the universal association of enzymes with living matter and the important part played by them in all types of vital activity. The activities of enzymes are especially prominent in the phenomena of digestion in animals, in the transformations of starch in sprouting seeds, etc These and many other observations lead to the view that enzymes are the essential organic catalysts, possessed or produced by all living cells, without which the processes of life would cease or be impossible. It is, furthermore, well established that these enzymes are of two types, called "exoenzymes" and "endoenzymes," according to their sphere of activity outside or within the confines of the cells that elaborate them. Both types are extremely important Exoenzymes, liberated by the manufacturing cells, penetrate and break down the organic materials

outside the cell, such as the proteins, **starches**, and fats of food materials, giving rise to soluble derivatives and so making it possible for the products of their activity to be absorbed through the cell membrane. Energy liberated by the enzyme action outside the cell is of comparatively little value to the cell. In general it is found that exoenzymes liberate relatively little energy, as heat, especially in the most commonly produced hydrolytic processes.

The endoenzymes elaborated and retained within the living cell, on the other hand, behave quite differently. The food substances, having been absorbed into the cell, may be further transformed and broken down by the action of the endoenzymes, and this process is accompanied by the liberation of relatively large amounts of energy, this energy being available to the cell.

The microorganisms of fermentation are notable for their ability to produce enzymes of both these classes. Molds, yeasts, and bacteria can secrete or elaborate a wide variety of enzymes, possibly of a greater range than any other single cells, since all the functions of growth, reproduction, digestion, assimilation, etc., that are distributed among the various tissues and organs of higher plants and animals seem here to be concentrated in the single minute cell. This may explain why it is that, in these low groups of extremely small plants which are essentially unicellular in structure, we find a higher degree of enzyme productivity and of fermentative capacity than is exhibited elsewhere in the world of living things.

The enzymes are therefore the reactive substances or catalyzers that microorganisms employ in bringing about the specific changes or fermentations that are characteristic of different species or groups of microbes One can visualize each strain, species, or genus as having its own armamentarium of enzymes or its own peculiar ability to secrete them In this individual potentiality may lie one of the factors that account for the variations in strains mentioned earlier in this chapter

TABLE 1.—ENERGY LIBERATED FROM ONE GRAM OF SUBSTRATE BY ENZYMES[1]

Exoenzymes	Calories	Endoenzymes	Calories
Pepsin	0	Lactacidase	82
Trypsin	0	Alcoholase	119 5
Rennet	0	Urease	239
Lipase	4	Oxidase (vinegar)	2,530
Invertase	9 3		
Maltase	10		
Lactase	23		

[1] ANDERSON, C. G., "An Introduction to Bacteriological Chemistry," Williams, Wood & Company The Williams & Wilkins Company Baltimore 1938.

Energy Relationships.—The preceding table, which shows the energy liberated from 1 g. of substrate by enzymes, will give some insight into the relative energy values of exoenzymes and endoenzymes.

The question naturally arises, does the microorganism have a requirement for a major portion of the large amounts of energy liberated within the cell, or is much of the energy liberated merely due to the great excess of enzyme product or the result of unrestricted enzyme action? Experiments with yeast cells indicate rather emphatically that the energy evolved is not a measure of metabolic requirements, but is the result of enzymes acting on an abundant and suitable substrate. However, further experimentation is necessary to lend additional weight to this suggestion. Whatever the theoretical aspects, it is known in practice that under the artificial conditions of a great volume of fermenting liquid, in which billions of cells are active, energy in the form of heat may be liberated to a marked degree. In such large masses of fermenting material where the heat produced cannot be readily diffused or conducted away the rise of temperature may become so great as to impair the organisms that produce the reacting catalyst. In vinegar fermentation the heat energy evolved may be sufficient to cause a rise of temperature that may inhibit normal cell growth and stop further activity.

Intense Activity of Microorganisms.—Many microorganisms, in comparison to the higher organisms, are intensely active in respect to the chemical changes that they bring about. The examples cited above show this fact and also are typical of the fact that all fermentative processes are exothermic. Burchard[1] has calculated that 1 g. (wet weight) of *Micrococcus ureae* decomposes 180 to 1,200 g. of urea per hour, while Haacke estimated that 1 g. (wet weight) of a lactose fermenting organism breaks down 178 to 14,890 g. of lactose in 1 hr. Even though these calculations may be of only approximate value, the enormous activity of microorganisms is evident. This great chemical activity of microorganisms is associated with their simple life requirements, the ease with which they attack food for energy, the rapidity of growth or reproduction, and possibly their capacity for maintenance under different conditions.

Specific Types of Fermentative Change.—It has been intimated in the foregoing pages that the specific or characteristic kind of fermentation produced by an organism is dependent on its enzyme-producing powers One might expect, therefore, that those organisms most nearly related generically would be most similar in their fermentation relationships This seems to be generally the case, although it does not follow that organisms belonging to different biological groups are necessarily totally

[1] STEPHENSON, M., "Bacterial Metabolism," Longmans, Green & Company, London, 1930.

unlike in their ability to catalyze fermentation processes having some of the same end products. The "true" yeasts and some other budding fungi nearly related to them are the organisms that most commonly produce ethyl alcohol, and because of this fact it is often stated that the yeasts are the microorganisms of alcoholic fermentation, but there are many budding fungi, classified in groups or families morphologically very similar to yeasts, that apparently have no ability to produce ethyl alcoholic fermentation or any other with large quantities of a particular end product. On the other hand there are a few fungi, somewhat higher in the scale of organization than yeasts, that can produce ethyl alcohol under certain conditions of substrate, pH, relation to oxygen, etc. Even a very small number of species of bacteria can produce alcohol. These are unusual cases but apparently demonstrate the great versatility of the low colorless plants in developing their enzyme systems. In general, the molds that have marked fermentation power are producers of organic acids and of products of protein decomposition. Similarly, the fermenting types of bacteria commonly give rise to organic acids, frequently different from the acids produced by molds, and to higher alcohols than ethyl. It cannot be assumed that all species of microorganisms belonging to the three groups that have been mentioned as constituting the microorganisms of fermentation actually have marked ability in this respect. It would be more nearly correct to say that the significant fermentation organisms represent a minority of all the organisms so classed.

Energy Value of Substances.—The substances acted on by microorganisms present a dual role, the first as a food, the second as a material to be transformed through the action of enzymes that have been produced in excess of nutritional requirements. In general, the energy value of a substance, such as glucose, depends on the degree of oxidizability it can undergo. In the complete oxidation of glucose, as in burning, considerable energy is evolved. This is also true in the breakdown by aerobic organisms, while in the anaerobic breakdown of this sugar only a fraction of the potential energy is liberated. Consequently, in order to obtain an equivalent amount of energy, several times as much glucose must be broken down under anaerobic conditions as would be required under aerobic conditions.

The breakdown of nutrients in fermentation is not merely expressed in the evolution of heat but is in early stages at least always accompanied by division of new cells. For example, in the manufacture of compressed yeast, the nutrient medium is supplied not only with organic food but also with a very large amount of air. Aerobic oxidations are favored and under such conditions, along with the fulfillment of nitrogen and phosphate requirements, pH control, and the employment of a low concen-

tration of sugar, large yields of yeast cells are produced. Under essentially anaerobic conditions, such as would exist in the normal industrial fermentation for ethyl alcohol, much more sugar must be utilized to produce the same quantity of yeast cells, and a large amount of the organic matter of the substrate is converted to the characteristic end products, one of which, ethyl alcohol, is still comparatively high in potential energy.

It should now be clear that a fermentation process that may become of industrial significance is not a simple matter of inoculating organic matter with microbes that can derive food from it, but is instead the setting up of a system of biochemical reactions between a substrate and an organism that can partially decompose it in a special manner under controlled and workable conditions. The substrate must be abundant, inexpensive, and fairly high in potential energy. The organism must have ability to attack, by means of its enzymes, certain chemical groupings in the composition of the substrate and, by hydrolysis, oxidation or reduction, or other means, disrupt certain bonds in the substrate molecules and give rise to stable products under the imposed conditions Thus by a single reaction or a series of coincident or successive enzyme reactions carried out under definite conditions it may bring about eventually a desired product or group of products of lower potential energy.

CHAPTER II

THE YEASTS

The yeasts and other yeast-like organisms, often grouped together under the name *Blastomycetes* or "budding fungi," belong to the subdivision of the thallophytes designated as the *Eumycetes*, or true fungi, since they possess no chlorophyll. All the organisms here grouped are unicellular plants of microscopic size and widely distributed in nature, and they occur especially in the top layers of the soil, in dust, and on the fruits and leaves of many plants The grape, apple, pear, and many other fruits have these organisms almost constantly present, and the soil of orchards and vineyards where the microbes live over the winter is particularly well populated with yeast cells. Distribution is easily effected by wind and on the bodies of bees, wasps, and other insects.

The budding fungi may be separated into two rather unequal divisions. the spore-forming (sporogenous) or true yeasts represented by the family *Endomycetaceae* (*Saccharomycetaceae*), and the nonsporing (asporogenous) pseudo or false yeasts, which are represented by the families *Rhodotorulaceae*, *Torulopsidaceae*, and *Nectaromycetaceae* The true yeasts include about 17 genera and a large number of so-called "species," many of which in turn show numerous slightly different strains or "types " Although most taxonomists are in agreement as to the main groups, definite classification into genera and species is externally difficult and unsatisfactory from the botanical standpoint. Industrially, however, the one genus *Saccharomyces* is of outstanding interest, as most yeasts having any technical uses belong herein.

The yeasts grow most luxuriantly in solutions containing sugar and the other necessary food requirements. In a fluid nutrient medium the cells ordinarily occur singly or in twos or threes or attached to one another in small clusters that are actually groups of cells of different generations, as a result of the characteristic method of vegetative increase by budding. On agitation, these groups break apart, and most of the cells eventually settle to the bottom of the container as sedimentary cells. If the culture medium remains undisturbed, islands of cells or films of greater or less complexity may appear on the surface. Sometimes these superficial cells may appear as chains or filaments of cells, resembling hyphae, and often with a number of smaller cells budded off

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at the nodes. Some true yeasts, such as those of the genus *Endomyces*,
may form a true mycelium or mass of cells. On favorable solid media,
compact colonies are produced, varying in size and surface markings
according to the nature of the substrate and the age of the colony.

Shapes of Cells.—The individual yeast cells are usually spherical,
ovoid, or ellipsoid in form. Broadly egg-shaped and elongated sausage-
shaped cells may be produced characteristically by certain yeasts,
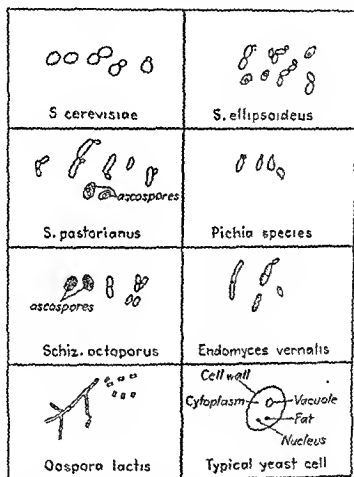
Fig. 1.—Some different types of yeast cells.

nevertheless, the shape of the active cell is not an exact means of species
identification, nor is variety in form in the same culture a proof of con-
tamination. Yeasts possess no flagella, and consequently the individual
cells are nonmotile. The accompanying figure illustrates a few of the
different types of yeast that occur in nature.

Size.—Yeast cells may vary considerably in dimensions, depending
on the species, nutrition, age, and other factors. The cell may vary
from 1 to 5 or more microns in width and from 1 to 10 or more microns
in length. With the approximately spherical cells of industrial yeasts a

diameter of 4 to 6 microns is probably a fair average, but great variations may occur even in the same culture. Most of the yeasts of extremely small size are of no present industrial importance except as they occur as contaminants.

In general, yeast cells are much larger than bacterial cells and could not be mistaken for the latter when observed microscopically. There are exceptions, however; some large bacteria exceed in size the smallest known yeasts mentioned in the figures given.

The Cell Wall.—A transparent permeable wall surrounds each cell or mass of protoplasmic material. The exact composition of the cell wall is not known. It is believed to be composed of "fungus cellulose," which differs in its chemical character from the cellulose complexes constituting the walls of green plants. The cell-wall membrane may be invisible or very thin in young cells but becomes thickened in old cells.

Contents of the Cell.—The protoplasm appears microscopically as composed of a grayish, finely granular, semifluid mass. Presumably it is of albuminous material intimately mixed with a "cell sap" of water with organic materials and salts in solution. Within the protoplast there is a nucleus and one or more rounded structures known as "vacuoles," which were originally so called because they appeared as clear or empty spaces but are now known to be the seat of reserve foods and finely divided "metachromatic granules" or volutin. The vacuoles are not conspicuous in very young cells but appear prominently in mature or old cells, and their nature can be somewhat determined by special staining methods. Generally a large vacuole is located near the nucleus, or there may be two or even more with a general polar location. The nucleus exists as a small mass near the center of the cell and cannot generally be seen without employing a special staining procedure. Iron hematoxylin is probably the best stain for this purpose. With this treatment the nucleus sometimes appears as a fairly compact body, but more often as a diffuse cluster of granules. The nucleus assumes an important role in reproduction. When budding takes place the nucleus moves toward the pole, and a portion of these granules (possibly half of them) migrates into the newly developing daughter cell.

Within the yeast cell protoplasm are numerous other substances or reserve materials in the form of droplets or granules, some of which are exceedingly complex in nature. In old cultures, some cells sometimes become thick walled and much enlarged and densely packed with these reserves. Such cells are sometimes called "durable" or "durative" cells, as they seem to have unusual resistance to adverse conditions.

These stored foods—including some carbohydrates (not starch), fats, or oils as refractile droplets, and some granules supposed to be proteins—
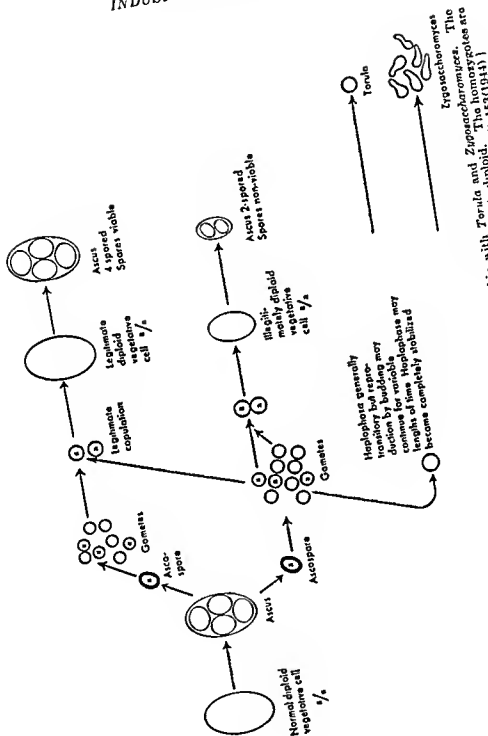
Fig. 3.—Life cycle of *Saccharomyces cerevisiae* showing the relationship with *Torula* and *Zygosaccharomyces*. The homozygotes are called illegiti[?], ni[?]ploids. The ascospores belong to two mating types a and α, and the heterozygote is the legitimate diploid. *[Courtesy of C. C. Lindegren, Wallerstein Labs. Commun., 7 (No. 22):153(1944)]*

In the case of *Saccharomyces cerevisiae*, there are believed to be two mating types, *a* and *α*. If two gametes of mating type *a* or two gametes of mating type *α* fuse, the zygote will contain the diploid number of chromosomes *aa* or *αα*. However, if the *a* type fuses with the *α* type, the resultant cell will be a legitimate diploid *a/α* (refer to Fig. 3).

According to Lindegren, most bakers' and brewers' yeasts (strains of *S. cerevisiae*) are illegitimately diploid. Illegitimate diploids usually form fewer viable ascospores [1]. These homozygous illegitimate diploids are fairly stable, more so than the heterozygous legitimate diploids, because they do not usually form ascospores and because they are homozygous. The legitimate diploids are stable as long as they reproduce vegetatively (by budding). However, sporulation may lead to a heterogamous mixture. Legitimate diploids produce 4 ascospores and occur commonly in nature.

In the case of *S. cerevisiae*, haploid cells differ from diploid cells in several respects. In general, the haploid cells are smaller, rounder, and rather variable. Such cells do not usually sporulate and when they do, the spores usually degenerate. The cells tend to grow in clusters. Colonies from such cells are usually rough, small, and variable. Diploid cells are generally larger and give rise to relatively large, smooth, and uniform colonies. [1]

A zygote may be formed from the fusion of two ascospores (endospores), of an ascospore and a vegetative haploid cell, or two vegetative haploid cells, of two sister haploid cells, or mother and daughter haploid cells, etc.

According to Lindegren, yeasts may be classified into two groups on the basis of their sexual behavior. In the first group, he places the genera *Schizosaccharomyces*, *Zygosaccharomyces*, *Zygopichia*, *Debaryomyces*, *Nadsonia*, and *Nematospora*. The vegetative cells of species of these genera are haploid. Following nuclear fusion, the diploid stage exists for a brief time but is followed by meiosis. The ascospores are haploid and give rise to haploid cells. In the second group, Lindegren places the genera *Saccharomyces*, *Saccharomycodes*, and *Hansenula*. Here the vegetative cells are diploid and the ascospores are haploid. Meiosis takes place during sporulation. Haploid vegetative cells, usually small and lacking vigor, may arise from haploid spores. These spores or vegetative cells formed from them may fuse and produce diploid cells, which are vigorous.

The breeding of yeasts has large possibilities. Winge and Laustsen produced a new hybrid by breeding together bakers' yeast and *Saccharo-*

myces validus. The resultant hybrid was an improvement over the standard bakers' yeast. A hybrid produced by mating haplophase cultures of *S. cerevisiae* with haplophase cultures of *S. carlsbergensis* was able to synthesize biotin, pantothenic acid, and pyridoxine, vitamins of the B-complex, in large amounts, according to Lindegren and Lindegren.[1,2] *S. cerevisiae* was unable to synthesize either biotin or pantothenic acid but able to synthesize pyridoxine. *S. carlsbergensis* was able to synthesize both biotin and pantothenic acid in large amounts but unable to synthesize pyridoxine.

The subject of genetics, as related to yeasts, is a large and growing one. The interested reader is referred to the end of this chapter for a list of references. The papers by the Lindegrens and Spiegelman in this country and by Winge and Laustsen of the Carlsberg Laboratory, Copenhagen, are particularly significant.

Yeast Spores.—Sporulation in yeasts is important for two reasons: it is the basis for a method of reproduction, and it serves an important role in maintaining the viability of yeasts during adverse changes in the environment   The ascospores of yeasts are more resistant to heat and desiccation than the vegetative cells, yet they are much less resistant to heat than bacterial spores

Sporulation.—Sporulation may be initiated by a deficiency in the food supply and the accumulation of toxic end products[3] but will not proceed unless certain other conditions are favorable, namely, the yeast cells must be young and vigorous; there must be plenty of air and moisture available; the pH of the medium must be suitable; inhibitory substances must be absent; and the temperature of incubation must be satisfactory. The presence of stimulating substances promotes sporulation.

In order to produce vigorous young cells, the yeast should be cultured in a suitable medium and transferred frequently. Reserve foods—glycogen, fat, and other products—are stored in the yeast. The use of such cells will insure sporulation, provided the other conditions are satisfactory and the yeast has the ability to form endospores. Oxygen is essential for sporulation; without it no spores are formed. Temperature is important. Each variety of yeast sporulates most readily in a given temperature range. Above certain temperatures and below others, sporulation fails to take place. Table 4 shows the maximum, minimum, and optimum temperatures for the sporulation of six varieties of yeasts studied by Hansen.

[1] LINDEGREN, C C, and G LINDEGREN, *Science,* **102** (No. 2637): 33–34 (1945).
[2] LINDEGREN, C C, *Missouri Botan Garden Bull* **34**: 37–43 (1946).
[3] GUILLIERMOND, A, "The Yeasts," translated and revised by F W. Tanner;
John Wiley & Sons, Inc., New York, 1920
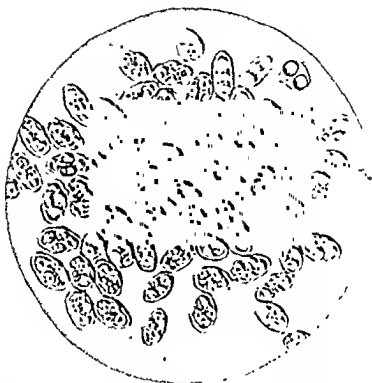
Fig. 4—Sporulation of yeast. (Courtesy of H. Kothe and F. R. Swift, The Fleischmann Laboratories.)

The optimum temperatures for the six varieties lie between 25 and 30°C., while three varieties have an optimum temperature of 25°C At the most favorable temperature for sporulation, ascospores begin to appear in 21 hr. or more.

Calcium sulphate stimulates sporulation but restrains budding Beer

TABLE 4—MAXIMUM, MINIMUM, AND OPTIMUM TEMPERATURES FOR SPORULATION OF CERTAIN YEASTS[1]

Yeast	Maximum temperature, degrees Centigrade	Minimum temperature, degrees Centigrade	Optimum temperature, degrees Centigrade
Saccharomyces cerevisiae	35 –37	9 –11	30
Saccharomyces pastorianus	29 –31 5	0 5– 4	27 5
Saccharomyces intermedius	27 –29	0 5– 4	25
Saccharomyces validus.....	27 –29	4 8– 5	25
Saccharomyces ellipsoideus	30 5–32 5	1 7– 5	25
Saccharomyces turbidans	33 –35	4 – 8	29

containing glucose or other sugars or gelatin stimulates sporulation, but ammonium salts have an adverse affect. Green, blue, violet, and ultra-violet rays have inhibitory effects on the formation of endospores. Extremes of pH definitely retard sporulation.

Methods of Inducing Sporulation.—There are a number of methods for inducing sporulation, some of which have been reviewed by Henrici[1] and by Lindegren and Lindegren.[2] They involve the use of nonnutrient, vegetable and/or fruit, and other types of substrates. Failure of a medium to induce sporulation in an unknown yeast does not necessarily mean that the yeast is asporogenous. Before arriving at such a conclusion, it may be necessary to try several different methods.

1. *Plaster of Paris Block.*—In this method, introduced by Engel, the upper surface of a block molded from pure plaster of Paris is scraped smooth. The block is placed in a glass container, which is fitted with a cover, and water is added until the block is about one-half submerged. The container, cover, and contents are then sterilized. Yeasts cells from a young, vigorous culture, which has been grown on a suitable medium with frequent transfer, are placed on the smooth surface of the block, and the container with contents is incubated at the desired temperature (25 to 30°C. usually) for 30 hr. or longer before observations are made. Hansen has devised a special flask for use in this method. Clay and blotting paper have been substituted for plaster of Paris.

2. *Plaster of Paris Slants.*[3] Plaster of Paris is mixed with an equal weight of water and introduced into bacterial culture tubes in appropriate amounts (about 10 ml.) The tubes are placed in a slanted position in a made by Graham and Hastings.[3] This is a modification and improvement drying oven at a temperature of approximately 50°C. and allowed to dry. Afterward the slants are plugged with cotton and sterilized. Lindegren and Lindegren, who used such slants in combination with their presporulation medium (to be described later), advocated the addition of 3 ml. of sterile water, acidified to a pH of 4 with acetic acid, to the base of each slant at the time of its use.

3. *Carrot Infusion Agar (McKelvey).*—Finely subdivided unpeeled carrots are autoclaved with water. The solids are separated from the extract by filtration and light pressure Sufficient agar is added to the extract to solidify it (about 20 g. per liter) Before adding the carrot infusion agar to the culture tubes, a small amount of calcium sulphate is placed in each. The medium is sterilized and slanted.

[1] HENRICI, A T., *Bact. Revs.*, **5**: 97–179 (1941).
[2] LINDEGREN, C C , and G. LINDEGREN, *Botan. Gaz*, **105** (No 3): 304–316 (1944)
[3] GRAHAM, V R , and F. E G. HASTINGS, *Can. Jour Res*, **19**: 251–256 (1941)

4. *Sporulation Stock Medium.*—Mrak, Phaff, and Douglas[1] have devised a medium which has produced good sporulation within 1 week or less with several hundred yeast cultures and which serves also as an excellent stock-culture medium. This medium is prepared by grinding equal weights of washed and unpeeled beets, carrots, cucumbers, and potatoes, mixing with a weight of water equal to the combined weights of the vegetables used, autoclaving the mixture for 10 min. at a pressure of 10 lb. per sq. in., separating the solids from the extract by filtering through cheesecloth and applying some pressure, adding 2 per cent of agar to the extract, distributing the infusion agar into culture tubes, sterilizing for 15 min. at a steam pressure of 15 lb., and slanting. The pH of the extract is reported to be about 5 7

5. *Presporulation Medium.*—Lindegren and Lindegren[2] have reported the achievement of good results through the combined use of a presporulation medium and plaster of Paris slants, although the presporulation medium itself will produce endospores if incubated for a few weeks. The presporulation medium devised by Lindegren and Lindegren contains the following ingredients

Beet leaves extract	10 ml
Beet roots extract	20 ml
Apricot juice	35 ml
Grape juice	16 5 ml
Yeast (dried)	2 g
Glycerol	2 5 ml
Calcium carbonate	1 g
Agar	3 g
Water to	100 ml

The medium is steamed for 10 min. and then distributed into culture tubes. The tubes are sterilized for 20 min. at 15 lb and cooled in a slanted position. If endospores are desired within a short time, the procedure is as follows: Approximately 1 ml of sterile water is pipetted or poured over the surface of a 3-day growth of the yeast on the presporulation medium. The tube is allowed to stand for 10 min and then a suspension of the yeast cells is made in the water This suspension is pipetted to the upper portion of a plaster of Paris slant About 3 ml of sterile water, acidified to a pH of 4 with acetic acid, are pipetted onto the bottom of the slant. The inoculated slants are incubated at 25°C for 1 to 2 days and then examined for *endospores*

[1] MRAK, E M, R V PHAFF, and H C DOUGLAS, *Science*, 96 (No 2197) 432 (1942)

[2] LINDEGREN and LINDEGREN, *loc cit*

6. *Vegetable Juice Medium.*—A medium prepared from a commercially available canned blend of eight vegetable juices with sufficient agar to solidify it has been used by Henrici[1] to obtain sporulation of yeasts. Wickerham, Flickinger, and Burton[2] have modified Henrici's medium and reported a "high degree of sporulation." The blend of eight vegetable juices from a can containing 1 pt. and 2 fl. oz. is adjusted to a pH of 6.8 with potassium hydroxide. One-half of a cake of compressed yeast is suspended in the juice and steamed for 10 min. to destroy the yeast cells and to liberate some of their contents.

The medium is again adjusted to pH 6.8 and then added to an equal volume of hot distilled water containing 4 per cent of melted agar. After mixing, the medium is dispensed into tubes or bottles and sterilized for 15 min at a steam pressure of 15 lb.

Not sooner than 8 hr. before use, the medium is freshly slanted. The whole surface of the slant is inoculated lightly with a 24-hr. culture of the yeast, which has been grown on a slant of yeast extract malt extract agar at about 28°C According to Wickerham and his associates, good sporulation of species of *Hansenula, Zygohansenula, Pichia,* and *Zygopichia* may be expected to take place within 3 days, of *Saccharomyces* and *Zygosaccharomyces* within 5 to 7 days, and of *Debaryomyces* within 5 to 20 days.

The junior author suggests that canned vegetable mixtures could also be used in the preparation of a sporulation medium. The vegetables could be used instead of fresh ones for the preparation of an infusion. The filtered extract would be solidified with agar.

7 *Other Vegetable and Fruit Substrates.*—Carrot plugs, potato plugs, cucumber wedges, grape juice, prune juice, raisins, cherry juice, raisin agar, and other substrates have been used successfully to induce sporulation of yeasts

8 *Gorodkowa's Medium*—Satisfactory results have been obtained by inoculating slants containing Gorodkowa's medium with an active culture of yeast.

Glucose	0 25 g.
Beef extract.	1 00 g
Agar or gelatin	1 00 g
Sodium chloride	0 50 g.
Distilled water	100 00 ml

9. *Kufferath's Medium.*—Malt is hydrolyzed with sulphuric acid and neutralized to various pH levels with calcium carbonate and sodium hydroxide. Agar is added, and the media are sterilized and inoculated.

[1] HENRICI, *loc cit.*
[2] WICKERHAM, L J., M H FLICKINGER, and K A BURTON, *Jour. Bact.,* **52** (No 5): 611–612 (1946)

10. *Soil Extract Gelatin Medium.*—This medium, developed by Niehaus (1932), is prepared by extracting 1,000 to 1,500 g. of garden soil with 1,000 ml. of water, by separating the solids from the extract by filtration, and by adding 15 per cent gelatin.

11. *Other Methods.*—Various other methods have been used for inducing yeasts to sporulate. Sterilized sausages have been used by Stelling-Dekker for sporulation of *Debaryomyces* species. Endospore formation may be occasionally induced in compressed yeast by storing it for 6 to 8 days in a refrigerator. Beechwood chips, soaked and sterilized, have been used by at least one investigator. Sugar solutions in shallow layers have been employed successfully.

The Isolation of a Single Spore.—Winge and Laustsen[1] regard the one-spore culture as being the smallest biological unit in yeasts instead of the single cell.
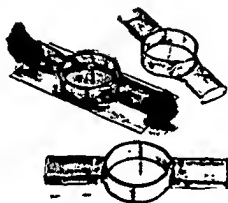
Fig. 5—Chamber used in isolating single spores. *(Courtesy of Dr. Ö. Winge, Carlsberg Laboratory, Copenhagen.)*

Laustsen has developed a technique for isolating all the spores of an ascus and cultivating them. His method is as follows[1] The yeast is caused to sporulate by placing it on a plaster block and incubating it for at least 30 hr. at room temperature. Using aseptic technique, a small drop of wort on a cover slip is inoculated with the spore material. The cover slip is then placed on a special operating chamber (Fig. 5).

An ascus is selected and withdrawn from the droplet, using a very fine glass needle. A small amount of wort naturally adheres to the ascus—more may be readily added if desired.

The isolation (Fig. 6) is carried out by means of two special glass needles. One needle, which has a point about 7 microns in diameter, is

introduced through one opening of the operating chamber (Fig. 5); the other needle, which has a point approximately 2.5 microns in diameter, is introduced through the other opening. The two needles are manipulated in such a manner as to burst the wall of the ascus against the surface of the cover slip. Each of the spores liberated is pulled into a separate droplet of sterile wort, which has been placed previously on the cover slip.

After the spore has germinated and a colony of cells has developed, some of the cells are transferred by the use of a sterile platinum needle to a Freudenreich flask containing sterile wort. Some of the remaining
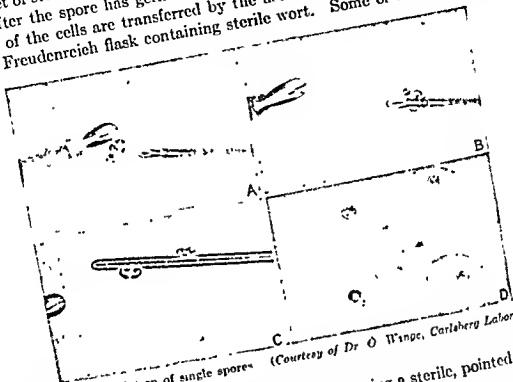
Fig. 6—The isolation of single spores   (Courtesy of Dr O Winge, Carlsberg Laboratory, Copenhagen)

cells may be transferred to a second flask by using a sterile, pointed piece of filter paper.

Occurrence and Distribution of Yeasts.—The budding fungi are of wide distribution in nature   Their habitats may include not only the upper layers of the soil but also many forms of organic matter, especially of plant origin, where carbohydrates are of common occurrence   Yeasts may be isolated particularly from the soil of vineyards and orchards; from the surfaces of grapes, apples, and most sweet fruits; from citrus fruits; and from the leaves and other parts of plants. They are carried into the air with dust and on the bodies of insects and thus may be widely disseminated. Proctor[1] has shown that yeasts may be found in the air at high altitudes. Some types of yeasts are also found occasionally on animal products.

    [1] PROCTOR, B E, *Proc. Am. Acad. Arts Sci.*, 69: 315 (1934).

Methods of Isolating Pure Cultures of Yeast.—In order to make sure that the culture isolated is pure and that it is not in reality a mixed culture of two morphologically related varieties of yeast or an undesirable combination of microorganisms, it is necessary to isolate single cells by special technique and to observe these cells during reproduction. Although there are several methods by which pure cultures may be isolated, the methods cited here will be restricted to the more important ones. Skill and patience are required in some of the methods of single-cell isolation.

1. *Moist-chamber Method of Hansen.*[1]—The chamber (Fig. 7) consists of a glass slide, a glass ring, and a cover slip with numbered squares (usually 16) The cover slip is attached to the ring by glass cement,
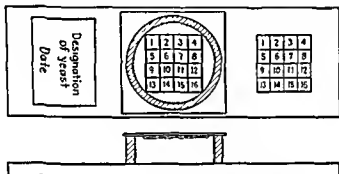
Fig 7 —Moist chamber for Hansen method

wax, or a vaseline-wax mixture Before using the chamber, the component parts should be sterilized by flaming them carefully

The culture of yeast from which the single-cell isolation is desired is diluted with sterile beer wort or water until a drop contains only a few cells A drop of the diluted culture is then placed in a tube of sterile melted wort gelatin, and the tube is thoroughly agitated to distribute the yeast cells uniformly throughout the medium. One drop of this medium is spread out on a glass slide, and, using the 100 × magnification of a microscope, is examined for the approximate number of cells If the number is satisfactory (20 or less), a small drop of the liquid wort gelatin containing the cells is placed on the underside of the cover slip and spread thinly and uniformly over the numbered squares The chamber is placed so that the gelatin rests in an even layer on the cover slip until it has solidified. Afterward the chamber is inverted, a drop of sterile water is placed on the slide to ensure a moist atmosphere in the chamber, and the ring is sealed tightly to the slide by means of vaseline

[1] JÖRGENSEN, A., "Practical Management of Pure Yeast," revised by A Hansen, Charles Griffin & Company, Ltd , London, 1936

The single cells are then located microscopically, and their positions are mapped on a diagram corresponding to the marked cover slip. Several moist chambers are usually prepared at the same time. They are then incubated at room temperature or around 25°C. for 2 or more days, during which time the growing yeast cells are carefully observed. During this time the single cells will have developed into colonies. Tubes of sterile wort are then inoculated from the individual colonies known to have grown from single cells.

2 *The Lindner Method.*—This method is a modification of the Hansen procedure. The culture containing the desired yeast is diluted in sterile wort, cider, or grape juice. Using a sterile crow-quill pen or wire, five rows of droplets, 5 droplets to a row, are deposited on a sterile cover slip that may or may not be marked into numbered squares. The cover slip is placed, culture side down, over the concavity of a sterile hollow ground-glass slide or ring slide after placing a drop of sterile water in the chamber. It is sealed with vaseline or paraffin. The droplets are examined microscopically, and those containing a single cell are marked by drawing small circles around them, or, if the squares are numbered, the locations are recorded by sketching the position of the droplet on a corresponding drawing After incubation and observation, the cultures are transferred to sterile wort.

3. *Dilution-plate Method.*—Tubes of dextrose, malt, or other suitable agar are melted and cooled to 42 to 44°C. Using a loop, a tube of agar is inoculated with the yeast-containing culture. The tube is thoroughly shaken, a unit amount of this medium is transferred to a second tube of melted agar. A third tube is inoculated in the same manner from the second tube. The contents of each of the three tubes are poured into petri dishes, and the plates incubated at 25 to 30°C. for 2 or more days. When the colonies have developed, samples of those which appear to consist of pure yeasts are examined with the microscope, using hanging drops If the yeast appears to be pure and satisfactory, sterile wort may be inoculated from the colony. This method, though simple, does not ensure the isolation of a culture from a single cell.

4. *The Micromanipulator Method.*—The micromanipulator may be used successfully in isolating pure cultures of yeasts from single cells. In experienced hands, this method is rapid The euscope, attached to the microscope, aids in the isolation of single cells.

Giant Colonies.[1]—Lindner suggested that giant colonies may contribute information that would be useful in identifying a yeast. A large

[1] GUILLIERMOND, A , "Clef dichotomique pour la détermination des levures," Librairie Le François, Paris, 1928, "The Yeasts," translated and revised by F. W. Tanner, op cit

horizontal surface of a suitable nutrient agar or gelatin medium contained in an Erlenmeyer flask or a large bottle is inoculated at one point in the middle with the aid of an inoculating needle. Petri dishes may be used, but owing to their susceptibility to contamination, they must be wrapped with surgeon's tape or packed in sealed containers to prevent contamination. Incubation is usually made for a period of 1 to 2 months at or near 20°C. The use of moist chambers aids in preventing the agar from shrinking prematurely.

Identification of Yeasts.—Yeasts are identified on the basis of a large number of observations—morphological, physiological, biochemical, cultural, and others. The shape and size of the cell; the optimum, minimum, and maximum temperatures for budding, sporulation, and film formation; the copulation of cells; the morphological nature of the ascs and ascospores; the method in which ascospores germinate; the characteristics of the sediment formed in wort; the appearance of colonies grown on various solid media; the characteristics of giant colonies; and the biochemical characteristics, such as the action on various sugars, are some of the more important factors considered in identifying an unknown yeast. For an intensive review of this subject the reader is referred to the works of Hansen, Lindner, Guilliermond, Tanner, Kufferath, and others.

The Nutrition of Yeasts.—Yeasts, like bacteria and other forms of life, require certain food materials and environmental conditions for proper growth and reproduction. Some elements are basically necessary as, for example, carbon, hydrogen, oxygen, nitrogen, phosphorous, potassium, sulphur, calcium, iron, and magnesium. There is accumulating evidence which indicates that trace elements also play an important role in nutrition. Vitamins and/or other organic compounds are required for the satisfactory development or functioning of most types of yeasts. An adequate supply of water is essential for carrying out their life activities.

Carbon.—In considering sugars as carbon sources, one must be reminded that the ability of a yeast to assimilate a sugar may be quite different from its ability to ferment the same sugar. Likewise the ability to assimilate a given compound varies with different varieties of yeasts.

Carbon may be supplied in the form of sugars, aldehydes, salts of some organic acids, glycerol, or ethanol.

Guilliermond has stated that impure maltose is best suited to the metabolism of yeasts.[1] Sucrose, glucose, fructose, and raffinose are not so important from the viewpoint of assimilation, yet some of these sugars are

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[1] GUILLIERMOND, A., "The Yeasts," translated by F. W. Tanner, John Wiley & Sons, Inc., New York, 1920.

readily fermented. Lactose is assimilated only in certain isolated cases

Acetates, citrates, lactates, malates, succinates, and tartrates, as well as lactic, malic, succinic, and tartaric acids, may be used as sources of carbon

Ethyl alcohol, in low concentration, occasionally may be utilized.

Nitrogen.—This element may be supplied to yeasts, depending upon the species or strain, in the form of ammonia, ammonium salts, amino acids, peptides, peptones (or other soluble protein derivative), nitrates, or urea. However, the last two are not usually very satisfactory sources of nitrogen for most yeasts

Ammonia and ammonium salts, particularly ammonium sulphate appear to be most suitable sources of nitrogen on account of their availability, low cost, and ready assimilation. Ammonia is apparently preferred by yeasts to other sources of nitrogen when these are present in a medium Amino acids are deaminized and the ammonia used by the yeasts.

Wort, which contains amino acids and other soluble nitrogen compounds (produced by the action of the proteolytic enzymes of malt on the proteins of barley), is an adequate and good source of nitrogen (refer to the chapter on Brewing).

UTILIZATION OF AMINO ACIDS.—There is considerable difference among the amino acids in respect to their availability and value as nitrogen sources for yeasts. The difference depends in part upon the position of where the amino group present and the isomeric form available. In most cases the amino group is attached to the carbon atom adjacent to the carboxyl group (alpha position), the nitrogen from the amino group is utilized For example, aspartic acid, glutamic acid, leucine, asparagine, glycine, and tyrosine are very good sources of nitrogen for yeasts. According to Nielsen,[1] 99 per cent of the nitrogen from aspartic acid, asparagine, and glycine is assimilated by yeasts. The structural formulas of these compounds follow:

COOH
CHNH₂
CH₂
COOH

Aspartic acid

COOH
CHNH₂
CH₂
CH₂
COOH

Glutamic acid

CH₃ CH
CH₂
CHNH₂
COOH

Leucine

COOH
CHNH₂
CH₂
CONH₂
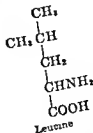
Asparagine

[1] NIELSEN, N., *Compt. rend. trav. lab. Carlsberg, Sér. physiol.*, 21: 395 (1936); *Sér. chim.*, 22: 384 (1937)

CH₂·NH₂ ... (chemical structure: Glycine)

(chemical structure: Tyrosine)

CH_2NH_2
$COOH$
Glycine

HOC ... C CH₂ CHNH₂ COOH
Tyrosine
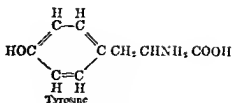
In some instances only a fraction of the total nitrogen is utilized. Only about one-half (56 per cent) of the nitrogen of tryptophane and one-third (34 per cent) of the nitrogen of histidine are used, indicating that the alpha-amino group is attacked [1]

(chemical structure: l-Tryptophane)

(chemical structure: l-Histidine)
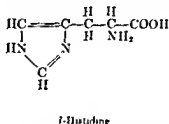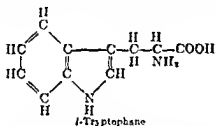
All amino acids except glycine exist in two isomeric forms (the dextro and levo forms).[1] Yeasts appear to be able to utilize the form of amino acid that occurs naturally in preference to the synthetic isomer; but will utilize both isomers of some amino acids, for example, the dextro and levo forms of aspartic and glutamic acids and asparagine.

CONVERSION OF AMINO ACIDS TO ALCOHOLS.—Ehrlich has shown that certain amino acids, namely, leucine, isoleucine, phenylalanine, tryptophane, and tyrosine, are converted to alcohols by yeasts. This work has been confirmed by Thorne. The reaction is a hydrolytic deamination and decarboxylation. The amino group is hydrolyzed and the carboxyl group is decarboxylated in accordance with the following general reaction

$$R \ CHNH_2 \ COOH + H_2O \rightarrow R\text{-}CH_2OH + NH_3 + CO_2$$
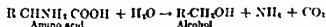
Amino acid ... Alcohol

The reactions for the conversion of certain amino acids to alcohols follow:

(chemical structure: Leucine → isoamyl alcohol)

$$CH_3\text{-}CH + H_2O \rightarrow CH_3\text{-}CH + NH_3 + CO_2$$

Leucine ... isoamyl alcohol
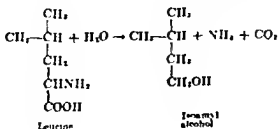
[1] BURK, D., and C. K. HORNER, Wallerstein Labs Commun., No. 6: 5–23 (1939)

$$\begin{array}{c} CH_2 \\ | \\ CH_2 \\ | \\ CH_3{-}CH \\ | \\ CHNH_2 \\ | \\ COOH \end{array} + H_2O \rightarrow \begin{array}{c} CH_3 \\ | \\ CH_2 \\ | \\ CH_3{-}CH \\ | \\ CH_2OH \end{array} + NH_3 + CO_2$$

Isoleucine   Amyl alcohol

Phenylalanine

$\rightarrow$ Phenylethyl alcohol

$\cdots CH_2 CHNH_2 COOH + H_2O$

$\rightarrow \cdots CH_2 CH_2OH + NH_3 + CO_2$

Tyrosine

$\cdots C.CH_2 CHNH_2 COOH + H_2O$

Tyrosol

$\rightarrow \cdots C CH_2 CH_2OH + NH_3 + CO_2$

Tryptophane

$\cdots C.CH_2 CHNH_2 COOH + H_2O$

Tryptophol

$\rightarrow \cdots C.CH_2 CH_2OH + NH_3 + CO_2$
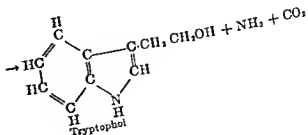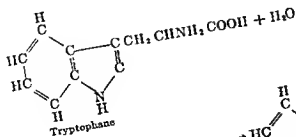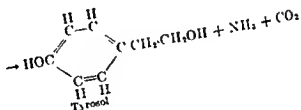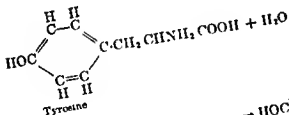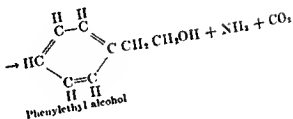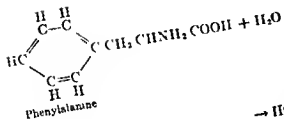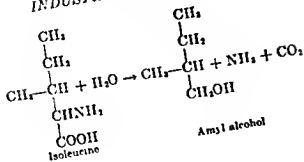
Thorne[1] has shown that isobutyl alcohol may be formed from valine by yeast in accordance with the following reaction:

$$\begin{array}{ccc} \text{CH}_3 & & \text{CH}_3 \\ | & & | \\ \text{CH CHNH}_2\text{ COOH} + \text{H}_2\text{O} \rightarrow & & \text{CH CH}_2\text{ OH} + \text{NH}_3 + \text{CO}_2 \\ | & & | \\ \text{CH}_3 & & \text{CH}_3 \\ \text{Valine} & & \text{isobutyl alcohol} \end{array}$$
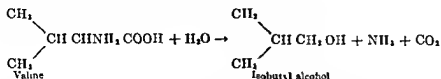
The ammonia liberated by any of the foregoing reactions is available for utilization by yeasts The reactions explain the origin of some of the constituents of fusel oil, such as amyl alcohol, isoamyl alcohol, and isobutyl alcohol.

EFFECT OF VITAMINS ON NITROGEN ASSIMILATION.—The ability or the inability of a given species of yeast to utilize a particular nitrogen source has been used as one means of classifying yeasts. Stelling-Dekker[2] employed potassium nitrate in classifying the sporogenous yeasts. Later, Lodder[3] used additionally ammonium sulphate, asparagine, peptone, and urea as aids in the classification of asporogenous yeasts. In order to determine the ability of the yeasts to utilize a particular nitrogen compound, she used a modification of the auxanographic method. A large inoculum of yeast was employed to supply the growth-promoting substances required.

Lodder found that the majority of yeasts examined by her were able to utilize the nitrogen-containing compounds tested, but that all the species of *Kloeckera* and some of those of *Torulopsis* were unable to utilize ammonium sulphate, asparagine, and urea.

Studies of the assimilation of nitrogen compounds by species of *Candida* have led to contradictory results, particularly in respect to the utilization of ammonium sulphate, asparagine, peptone, and urea.[4][5][6]

Wickerham[7] carried out tests with various species of yeasts reputedly unable to utilize ammonium sulphate, urea, and asparagine. He demon-

[1] THORNE, R, *Jour. Inst Brewing*, **43**: 288 (1937)

[2] STELLING-DEKKER, N M., Die Hefesammlung des Centraalbureau voor Schimmelcultures I Teil Die sporogenen Hefen, *Verhandel Koninkl Akad Wetenschap Amsterdam, Afdeel Natuurk*, Sec. II, Deel **28**: 1-517 (1931)

[3] LODDER, J, Die anaskosporogenen Hefen, Erste Hälfte, *Verhandel. Koninkl Akad. Wetenschap, Amsterdam, Afdeel Natuurk*, Sec II, **32**: 1-256 (1934)

[4] LANGERON, M and P. GUERRA, *Ann parasitol humaine et comparée*, **16**: 162-179 (1938).

[5] DIDDENS, H A, and J LODDER, Die Hefesammlung des Centraalbureau voor Schimmelcultures II Teil. Die anaskosporogenen Hefen, Zweite Hälfte, N V Noord-Hollandsche Uitgeversmaatschappij, Amsterdam, 1942

[6] MACKINNON, J E, and R C. ARTAGAVEYTIA-ALLENDE, *Jour Bact*, **49**: 317-333 (1945).

[7] WICKERHAM, L J, *Jour Bact*, **52**, (No 3). 293 (1946)

strated that the strains of the species tested (obtained from the Northern Regional Research Laboratory stock collection) were able to utilize all three of these nitrogen compounds when his special medium containing vitamins and certain trace elements was used. He also showed that urea in 0 092 per cent concentration inhibited the growth of all but 1 of the 10 species of *Candida* tested. However, urea in a concentration of 0.046 per cent concentration was assimilated by all 10 species.

The results obtained by Wickerham are of great significance, particularly in respect to the classification of yeasts, for they show that in the presence of vitamins, yeasts may assimilate nitrogen sources which they otherwise might not.

Wickerham devised and used the following medium (shown in Table 5), which includes trace elements and eight pure vitamins. This medium was used in both solid and liquid forms.

TABLE 5—COMPOSITION OF WICKERHAM'S SPECIAL MEDIUM

Ingredients	Concentration	Ingredients	Concentration
Trace elements		Vitamins	
Boron, as H₃BO₃	0 01 p p m	Biotin	2 µg *
Copper, as CuSO₄ 5H₂O	0 01 p p m	Calcium pantothenate	400
Iodine, as KI	0 10 p p m	Inositol	2,000
Iron, as FeCl₃ 6H₂O	0 05 p p m	Niacin	400
Zinc, as ZnSO₄ 7H₂O	0 07 p p m.	*p*-Aminobenzoic acid	200
		Pyridoxine hydrochloride	400
		Thiamin hydrochloride	400
		Riboflavin	200
		Carbon source	
		Glucose	10 0g*
Salts			
KH₂PO₄	0 875 g*	Nitrogen sources²	
K₂HPO₄	0 125 g	(NH₄)₂SO₄	1 00g*
MgSO₄ 7H₂O	0 50 g	KNO₃	0 78
NaCl	0 10 g	Urea	0 46
CaCl₂ 2H₂O	0 10 g	Asparagine	1 00
		Peptone	1 32

¹ WICKERHAM, L J, Jour Bact, 52 (No 3), 293 (1946).
² Used separately.
* Units given in terms of a liter of solution

Mineral Requirements.—The exact mineral requirements of yeasts in many cases are not fully known. The literature contains some conflicting statements on the subject, as has been shown by Joslyn¹ in his extensive

¹ JOSLYN, M A., *Wallerstein Labs Communs*, 4 (No. 11): 49 (1941).

review of the mineral metabolism of yeasts. Further careful research in this field should lead to a better understanding of the subject and practical improvements in the selection of media for growth, sporulation, and fermentation.

Chemical and spectroscopic analyses of yeasts and the media in which they are grown furnish considerable valuable information regarding the indispensability of the elements. However, the mere fact that an element is found in the ash of yeast in small amounts does not establish its essentiality.

The following table presents data concerning the composition of yeast ashes as determined by three different laboratories.

TABLE 6—COMPOSITION OF YEAST ASHES[1,2]

Ash constituent	Top yeast[3]	Bakers' yeast[4]	Bakers' yeast[5]
P₂O₅	52 3	52 3	54 5
K₂O	35 4	35 4	36 5
Na₂O	0 06	0 60	0 7
MgO	4 8	4 8	5 2
CaO	t 56	1 56	t 4
SiO₂	1 1	1 1	1 2
SO₃	0 41		0 5
Cl			Trace
FeO	0 43	0 34	Trace

[1] JOSLYN M A., Wallerstein Labs. Communs., 4 (No 11) 49 (1941)
[2] In percentage.
[3] FULMER et al (1928)
[4] HELLER (1925)
[5] FREY (1930)

A study of this table indicates that phosphorus and potassium are present in large amounts in yeast ash, comprising about 90 per cent of the total elements. The elements magnesium, calcium, silicon, sodium, iron, and sulphur are present in considerably smaller quantities.

Phosphorus is a particularly important element in the life processes of yeasts. It plays a very significant role in the production of ethyl alcohol from sugars as, for example, in the formation of hexose and triose phosphates. It is a component of cozymase and of cocarboxylase (an activator of the enzyme carboxylase). It is found in nucleic acid, in lecithin, and in other components of the yeast cell.

Natural media and worts normally contain sufficient of the inorganic elements for good growth. However, in studying the effect of a given element or growth-promoting substance on growth or fermentation, it is in most cases essential to know the exact composition of the medium employed. Ordinary chemical analysis often fails to reveal sufficient

ganese, are of considerable importance. Elvehjem[1] demonstrated that bakers' yeast will grow poorly in a medium low in iron and copper. These elements appear to be essential for the elaboration of cytochrome. Iron catalyzes respiration through the action of catalase, peroxidase, and cytochrome oxidases. According to Richards,[2] growth of yeast is stimulated when thallium is added to Williams' medium (20 g. of sucrose, 3 g. of $(NH_4)_2SO_4$, 2 g. of KH_2PO_4, 1.5 g. of asparagine, 0.25 g. of $CaCl_2$, and 0 25 g. of $MgSO_4 \cdot 7H_2O$ in 1 liter of distilled water). Edbacher is of the opinion that manganese activates yeast arginase.

Further information concerning the mineral metabolism of yeasts and methods of studying the nutrition of yeasts will be found in the following paragraphs.

TABLE 8.—SOME SEMISYNTHETIC MEDIA

Substance	Medium of Devereux and Tanner,[1] grams	Medium F of Fulmer and Nelson,[2] grams	Medium of Mayer,[3] grams
Sucrose	10 0	10 0	15 0*
Dextrin	.	0 60	
NH_4Cl	0 12	0 188	
K_2HPO_4	0 05	0 100	
KH_2PO_4	. .		0 1
$CaCl_2$	0 01	0 100	
$Ca_3(PO_4)_2$			0 1
$MgSO_4$	0 02		0 1
Distilled water	100 00 cc	100 0 cc.	100 0 cc.

[1] DEVEREUX, E D., and F W TANNER Jour Bact., 14: 317 (1927)
[2] Formula for incubation at 30°C   F W TANNER, E D, DEVEREUX, and F M HIGGINS, Jour Bact, 15: 45 (1928)
[3] GUILLIERMOND, A, "The Yeasts," translated and revised by F W TANNER, John Wiley & Sons, Inc., New York, 1920
* Candied sugar.

Media.—Food materials are supplied to yeasts in the form of media (singular, medium) which contain all the essential materials necessary for growth, reproduction, or fermentation. Media may be synthetic, semisynthetic, or nonsynthetic in nature   A synthetic medium is one in which the composition of all the ingredients is known. A semisynthetic medium is one containing some constituents of known composition and some whose nature is only approximately known. A nonsynthetic medium is one whose exact composition is not known. The composition of chemically pure salts, sugars, acids, and certain other chemical compounds is definitely established, whereas that of such items

[1] ELVEHJEM, C A, Jour Biol Chem, 90: 111 (1931)
[2] RICHARDS, O W, Jour. Am Chem Soc, 47: 1671 (1925)

as beef extract and peptone are more qualitative than quantitative. Media such as apple juice and grape juice are natural sources of the required food materials for the yeasts, as is evident by the fact that the yeasts found on the surfaces of apples and grapes rapidly ferment at optimum temperatures the juices expressed from them.

Media favorable for the growth of yeasts are also suitable for the growth of molds. Owing to the rather acid nature of these media many bacteria fail to develop well in them or are inhibited entirely.

For a further discussion of the subject of yeast nutrition, the reader is referred to Guilliermond's or other standard texts.

Relative Rates of Fermentation of Glucose and Fructose.—Not all sugars are fermented at the same rate. Nor do all yeasts act with equal efficiency. In low concentrations of sugar, fructose is fermented at a slower rate (expressed as milligrams of carbon dioxide per minute) than glucose by brewers' yeast at 30°C.[1] Although the maximum rates for the fermentation of these two sugars differ but little, the rate for glucose is slightly greater than that for fructose at the same concentration. Glucose is fermented at about the same rate in concentrations between 1 and 10 per cent; fructose, between 2 and 8 per cent. At high concentrations of the sugars, the rate of fermentation of fructose is less than that of glucose. This is probably referable to the difference in the ease with which the enzymes involved can bring about the cleavage of the sugar molecule.

Yeast Enzymes.—The enzymes present in yeast include, usually, at least three different groups: those concerned with respiration; those concerned with breaking sugars down to the hexose stage, for example, maltase and invertase; and finally those concerned with fermentation.

Enzymes catalyze the complex chemical changes that take place in nutrient sugar solutions. Whether or not a carbohydrate is fermented or assimilated depends on the nature of the enzymes elaborated by the yeast, provided, of course, that conditions are otherwise favorable for fermentation or growth. Polysaccharides, in general, are not fermented. However, Wickerham and associates[2] found that *Endomycopsis fibuliger* possessed an extracellular amylase system with a high alpha- to beta-amylase ratio. Lactose is fermented by *Saccharomyces fragilis* (*S. kefir*), the yeast of the fermented milk product, kefir, and by a few other yeasts but not by *S. cerevisiae* and *S. ellipsoideus*, which represent the best-known and probably most widely distributed types of yeast.

[1] HOPKINS, R. H., and R. H ROBERTS, Kinetics of Alcoholic Fermentation of Sugars by Brewer's Yeast, *Biochem Jour*, **29**: 931 (1935)

[2] WICKERHAM, L. J, L B LOCKWOOD, O. G. PETTIJOHN, and G. E. WARD, *Jour Bact.*, **48** (No. 4): 413 (1944).

The enzymes of yeasts are of two kinds: endoenzymes (intracellular) and exoenzymes (extracellular). These enzymes react according to the general laws governing enzyme reactions but show also a degree of specificity in each case.

The enzymes of yeasts may be classified as hydrolases and desmolases

Hydrolases are enzymes that convert carbohydrates, proteins, and esters to simpler substances by the addition of water followed usually by cleavage. For example, sucrose is converted, after the absorption of water, to 1 molecule each of glucose and fructose as a result of the action of sucrase, an enzyme; while raffinose, a trisaccharide, is converted to 1 molecule each of fructose, glucose, and galactose through the action of melibiase and sucrase, enzymes possessed by "bottom" yeast.

Glycogen is hydrolyzed to glucose, but no cleavage follows the addition of the molecule of water to the glycogen molecule. Although glycogenase may thus convert glycogen to glucose, it may, under certain conditions, cause the reaction to go in the opposite direction. Thus, some enzymes have the ability to catalyze reversible reactions and so to synthesize compounds as well as to break them down to simpler components.

Desmolases are enzymes or enzyme complexes involved in respiration and metabolism. Zymase is a desmolase of intracellular origin and rarely or never passes through the cell wall into the surrounding medium.

Zymase is not a single enzyme, but a complex of enzymes and coenzymes. The term "holozyme" has been coined to include the zymase complex plus all its activators. Hexokinase, oxidoreductase, enolase, carboxylase, and phosphatase are said to be some of the enzymes of this complex

Table 9 summarizes data concerning some of the enzymes found in yeasts, the substrates acted upon, and the end products formed as a result of this action.

Coenzymes and Activators.—A coenzyme, phosphate, magnesium, or some other substance may be essential in order that an enzyme may function properly. Cozymase (coenzyme I) is required for the action of oxidoreductase; cocarboxylase, for carboxylase, the enzyme which splits carbon dioxide from such alpha-ketonic acids as pyruvic acid Magnesium is essential for the activation of phosphatase.[1] Glutathione is required by methylglyoxalase

Cell-free yeast juice from crushed or autolyzed cells will ferment sugar but more slowly than yeast cells, a fact demonstrated by Buchner. If yeast juice is dialyzed by passing it through a semipermeable membrane, neither the dialyzate, the portion passing through the membrane,

[1] Tauber, H "Enzyme Chemistry," John Wiley & Sons, Inc , New York, 1937

Table 9.—Some Enzymes of Yeasts, Substrates Acted upon, and Products Formed

Enzyme	Substrate	Products formed
I Hydrolases:		
A Carbohydrases:		
1 Sucrase ...... (Saccharase, invertase, invertin)	$C_{12}H_{22}O_{11}$ Sucrose	$C_6H_{12}O_6 + C_6H_{12}O_6$ Glucose Fructose
2 Maltase . . .	$C_{12}H_{22}O_{11}$ Maltose	$2C_6H_{12}O_6$ Glucose
3 Lactase	$C_{12}H_{22}O_{11}$ Lactose	$C_6H_{12}O_6 + C_6H_{12}O_6$ Galactose Glucose
4. Melibiase, ...	$C_{12}H_{22}O_{11}$ Melibiose	$C_6H_{12}O_6 + C_6H_{12}O_6$ Galactose Glucose
5. Trehalase......	$C_{12}H_{22}O_{11}$ Trehalose	$2C_6H_{12}O_6$ Glucose
6. Glycogenase......	$(C_6H_{10}O_5)_x$ Glycogen or $C_6H_{12}O_6$ Glucose	$C_6H_{12}O_6$ Glucose $(C_6H_{10}O_5)_x + xH_2O$ Glycogen
B. Proteolytic enzymes:		
1. Proteases	Yeast proteins	Proteoses, peptones, and polypeptides
2 Peptidases..	Peptides	Amino acids
C. Esterases:		
1. Phosphatases:		
a. Polynucleotidase ...	Nucleic acid	Mononucleotides
b. Phosphatase ..	Hexose + H_3PO_4	Hexosephosphate
D. Amidases:		
1. Asparaginase...	$H_2N\,CO\,CH_2\,CHNH_2\,-COOH$ Asparagine	$HOOC\,CH_2\,CHNH_2\,-COOH + NH_3$ Aspartic acid
II. Desmolases:		
A. Zymase group:		
1. Oxydoreductase .. (Mutase, dehydrase)	$RCHO$ Aldehyde	$RCH_2OH + RCOOH$ Alcohol Acid
2. Glycerolphosphoric dehydrogenase	Glycerolphosphoric acid	Glyceraldehyde phosphoric acid
3. Carboxylase......	$CH_3\,CO\,COOH$ Pyruvic acid	$CH_3\cdot CHO + CO_2$ Acetaldehyde Carbon dioxide
4. Methylglyoxalase.	$CH_3CO\,CHO$ Methylglyoxal	$CH_3CHOHCOOH$ Lactic acid
5. Hexokinase	Hexoses	Active hexoses

nor the residue will produce fermentation of sugar. Should the two fractions be mixed, however, fermentation ensues.

The residue, the portion not passing through the semipermeable membrane, is thermolabile, being destroyed by boiling. The non-dialyzable enzymes are found in this fraction.

Cozymase.—The dialyzate is thermostable and contains, in addition to other substances, cozymase or coenzyme I.

Euler and Schlenk[1] have proposed the following structural formula for cozymase or coenzyme I

Cozymase (Coenzyme I)
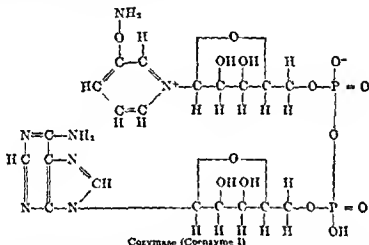
Euler and others believe that cozymase is a hydrogen-carrying coenzyme, which picks up 2 atoms of hydrogen (to form dihydrocozymase) and later donates them. It acts as an intermediate between two apodehydrases in the transportation of hydrogen from the donator system (glyceraldehyde phosphoric acid-phosphoglycene acid) to the acceptor system (acetaldehyde-ethyl alcohol) in the ethanol fermentation [2] (For further details concerning the function of cozymase, refer to page 157.)

Cozymase may be prepared by dialyzing fresh yeast juice, which contains about 0 5 g. of cozymase per kilogram of juice,[3] or by washing zymin with water.

The coenzyme may be isolated from most animal tissues; from many plants and fungi; from certain bacteria, namely, those giving rise to propionic and lactic acids; and from red blood corpuscles.

The Adenylic Acid System.—The dialyzate of yeast juice also contains a phosphate carrier system, which functions as a coenzyme. This

[1] Euler, H., The Chemistry and Metabolism of the Compounds of Phosphorus, Ann Rer Biochem, 7: 125 (1938)

[2] Euler, H von, Angew Chem, 50: 831 (1937)

[3] Meyerhof, O., und P Ohlmeyer, Biochem Zeit, 290: 334 (1937)

example, Stelling-Dekker used this test in classifying the genus *Saccharomyces*.

Raffinose is a trisaccharide that structurally is a combination of melibiose and sucrose with the glucose radical common to the two. Upon partial hydrolysis by sucrase it is converted to melibiose and fructose, the enzyme sucrase acting only on the sucrose portion of the trisaccharide. Melibiose (glucopyranose-6-α-galactopyranoside) is a disaccharide, which may be converted by the enzyme melibiase to glucose and galactose.
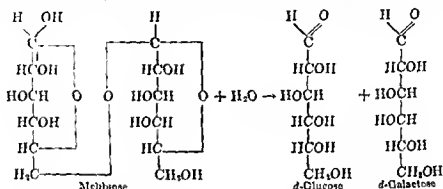
Yeasts, which secrete both sucrase and melibiase, hydrolyze raffinose to 1 molecule each of fructose, glucose, and galactose. Such yeasts completely ferment raffinose. Other yeasts, which possess sucrase but not melibiase, hydrolyze raffinose to melibiose and fructose. These yeasts ferment only fructose or one-third of the raffinose molecule.

A number of procedures have been devised to determine the extent to which raffinose is fermented. These involve the use of a quantitative apparatus, such as the van Iterson-Kluyver apparatus;[1] the absorption of the carbon dioxide produced during fermentation by means of barium hydroxide and the titration of the excess alkali with a standard acid solution;[2] the titration of the unfermented sugar after 14 days;[3] and a method devised by Wickerham,[4] which is described below.

In Wickerham's procedure, a 4 per cent nutrient solution of raffinose is dispensed in special tubes and inoculated with the yeast being tested. After the fermentation has subsided and the volume of gas has commenced to decrease, each tube is inoculated with a melibiose-fermenting yeast. A record is made of the amount of gas produced by each yeast.

The basal medium contained 0.45 per cent Difco yeast extract, 0.75 per cent peptone, and sufficient brom thymol blue to produce a green color

[1] Henrici, A. T., *Bact. Revs.*, **5**: 97 (1911).

[2] "Manual of Methods for Pure Culture Study of Bacteria," Leaflet VI, Society of American Bacteriologists, Geneva, N. Y., 1942.

[3] Zimmermann, J. G., *Zentr. Bakt. Parasitenk.*, II, **98**: 36 (1938).

[4] Wickerham, L. J., *Jour. Bact.*, **46** (No. 6): 501 (1943).

EREMASCOIDEAE
Mycelium
Cell division
Iso copulation
Oxidative

NEMATOSPOROIDEAE
Mycelium and budding cells, iso copulation; needle or spindle-shaped spores, with or without appendages. Oxid or ferm

Eremascus
Oxid
Mycelium
Cell division-no budding
Iso copulation
No orthospores.

Coccidiascus
mycelium
ation
-needle

Nematospora
Oxid & Ferm.
Oval long, irregular or mycelium-like cells
No apparent conjug.
4 or more spindle-shaped spores with appendage

Coccidiascus
Oxid
Round to oval cells
Iso copulation
8 spindle shaped spores

Saccharomyces
Ferm.
Round, oval to long cell shaped ascus
Pseudomycelium of conjug tubes times.
Iso or before copulat warty bordered times.
son or between haploid cells.
Spores round, kidney or hat shaped.

CYCLES CHARACTERISTIC OF TORULOPSIS
PULCHERRIMA LINDNA TROPICALIS AND
TAENNINA DEFORMANS

THICK-WALLED CELL WITH OXID
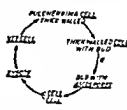
NOTE:
R = MEIOTIC OR REDUCTION DIVISION
P = PLASMOGAMY FUSION OF PROTOPLAST OF
    COPULATING CELLS
C = CARYOGAMY FUSION OF NUCLEI OF
    COPULATING CELLS
SINGLE UNDERLINE, ———→ HAPLOID GENERATION
DOUBLE UNDERLINE, ———→ DIPLOID GENERATION

at a pH of 7.0. Two-milliliter portions of this medium were added to each of a series of culture tubes measuring 12 by 150 mm. and containing inverted vials measuring 6 by 50 mm. The tubes and their contents were sterilized and to each tube was added aseptically 1 ml. of a 12 per cent solution of raffinose, sterilized for 20 min. at 12 lb. steam pressure. This medium was then inoculated with a young culture of the yeast being tested, which had been grown on a yeast extract agar slant. The tubes were incubated at 30°C. Gas production was recorded from time to time, and when the amount of gas in the inverted vial commenced to decrease, the tube was inoculated with a young culture of *S. carlsbergensis* NRRL 379, an active fermenter of melibiose. The production of additional gas indicated that the test yeast had not fermented melibiose. However, failure of *S. carlsbergensis* to produce additional gas was taken as evidence that the test yeast had already fermented melibiose.

Wickerham, in surveying 200 strains of industrial yeasts, found that the average length of time required for a yeast to complete the fermentation and for the volume of the gas entrapped in the inverted vial to commence to decrease was 7 days and that the longest time required was 16 days.

Henrici[1] used culture tubes of 25 by 100 mm. size containing 20 ml. of medium and inverted Wassermann tubes (10 by 75 mm.) as gas traps in carrying out the melibiose fermentation test. He suggested that tubes inoculated with pellicle-forming yeasts be shaken a day or two after inoculation in order that some of the cells might sink to the bottom, also that all tubes be shaken before reading the final results.

Auxanographic Techalque.—In order to determine whether or not a yeast is able to utilize a given sugar, Beijerinck[2] devised an auxanographic procedure. The basal medium, which contained 0.1 per cent potassium dihydrogen phosphate, 0.05 per cent magnesium sulphate, 0.05 per cent ammonium sulphate, and 2.0 per cent of washed agar, was prepared, sterilized, cooled, seeded heavily with the yeast being tested while still in the melted condition, and poured into petri dishes. The heavy suspension of yeast was used in order to provide the required growth-promoting substances. Small amounts of the sugars to be tested (in the dry form) were placed upon the dry surface of the solidified agar, sufficiently far apart to prevent mixing of the dissolved materials. Glucose was used as a control, since all yeasts are able to utilize it. The petri dishes were incubated at an optimum temperature for growth, usually 25 to 28°C. The sugars diffuse into the agar and, if they are assimilated, cause growth

[1] Henrici, *loc. cit.*

[2] Beijerinck, M. W., *Arch. néerland. sci.*, **23:** 367–372 (1889).

of the yeasts. No growth takes place when a sugar cannot be utilized. This procedure has been used by Lodder[1] and others.

Lodder[1] used a modification of Beijerinck's auxanographic technique in order to determine the extent to which certain nitrogen compounds were used by yeasts. The basal medium in this case contained 2 per cent glucose, 0.1 per cent potassium dihydrogen phosphate, 0.05 per cent magnesium sulphate, and 2 per cent of washed agar. In carrying out the test, approximately 2 ml. of a heavy suspension of the yeast being examined were placed in each petri dish and the basal medium added. The plates were incubated at 30°C. for a number of hours in order to dry the surface of the agar. Small amounts of the nitrogen compounds (ammonium sulphate, asparagine, potassium nitrate, peptone, and urea) were then placed on the surface of the agar. The petri dishes were incubated at 25°C., and later observed to determine which sources of nitrogen supported growth.

Since the auxanographic method may produce erroneous results if a sugar or nitrogen compound diffuses over too wide an area in the petri dish, some workers have preferred to use liquid media and to test for the utilization of each given substance in separate culture tubes.

GROWTH-PROMOTING SUBSTANCES

Definition.—Growth-promoting substances, or growth-accessory factors, may be considered to be substances which, when added to a medium containing the usual sources of energy, carbon, nitrogen, and basic inorganic salts, accelerate the growth of a microorganism.

Controversial Nature.—The subject of growth-promoting substances, especially bios, has been a cause of controversy, particularly during the first part of the present century. Failure to agree on findings in different laboratories has been due in large part to the use of different species or strains of yeasts or other microorganisms. Copping,[2] Williams,[3] and others have shown that the requirement for bios, for example, depends on the type of yeast and the composition of the medium.

Bios.—Space does not permit an extensive review of the literature concerning bios. The subject was reviewed, however, by Tanner,[4] in

[1] LODDER, J , Die Hefesammlung des Centraalbureau voor Schimmelcultures, II Teil. Die anaskosporogenen Hefen, Erste Hälfte, Verhandel. Koninkl Akad Wetenschap. Amsterdam, Afdeel. Natuurk , Sec. II. Deel 32 : 1–256 (1934)

[2] COPPING, A M., Biochem. Jour., 23: 1050 (1929).

[3] WILLIAMS, R J , J. L. WILSON, and F. W. VON DER AHE, Jour. Am Chem. Soc, 49 : 227 (1927).

[4] TANNER, F. W., Chem. Rev., 1: 397 (1925)

1925; by Lutman[1] in 1929; by Buchanan and Fulmer[2] and by Miller[3] in 1930.

Since the appearance of these reviews, considerable new research has been carried out concerning the growth substances of yeasts, and the identities of several components of bios have been established.

In 1935, Thimann[4] outlined the more important advances in the knowledge of bios, while Koser and Saunders[5] (1938) presented an extensive review concerning bacterial accessory growth factors, which included a survey of yeasts and molds.

Origin of the Term.—Wildiers[6] (1901) proposed the name "bios" for the hypothetical organic substance of biological origin that stimulated the growth of yeasts. He based his evidence for the existence of bios on a series of observations made during his researches. He observed that a beer yeast, a strain of *Saccharomyces cerevisiae*, grew poorly on a synthetic medium that contained ammonia as the source of nitrogen, but that the addition of a small amount of organic material, such as beer wort, caused rapid growth of the yeast. Likewise small amounts of inoculum sometimes failed to produce growth in a medium, whereas relatively large quantities of inoculum produced rapid growth. The filtrate from boiled yeast produced the same effect as a large inoculum. Liebig's meat extract and other organic substances of biological origin stimulated growth.

Wildiers's ideas on bios were soon challenged, but they served as a basis for subsequent important research.

Chemistry of Bios.—The complex known as bios is soluble in water and in 80 per cent alcohol but insoluble in absolute alcohol and ether. It is relatively stable to heat and moderately so to acids but is destroyed by boiling in a 20 per cent solution of sulphuric acid. A boiling solution of sodium hydroxide, of concentration much in excess of 1 per cent, usually destroys bios. It is dialyzable through a semipermeable membrane.[7]

Lucas[8] demonstrated that bios may be separated into two fractions

[1] Lutman, B. F., "Microbiology," McGraw-Hill Book Company, Inc., New York, 1929.

[2] Buchanan, R. E., and E. I. Fulmer, "Physiology and Biochemistry of Bacteria," Vol. II, The Williams & Wilkins Company, Baltimore, 1930.

[3] Miller, W. L., *Jour. Chem. Education,* **7:** 263 (1930).

[4] Thimann, K. V., *Ann. Rev. Biochem.,* **4:** 515 (1935).

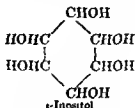[5] Koser, S. A., and F. Saunders, *Bact. Rev.,* **2:** 122 (1938).

[6] Wildiers, E., *Cellule,* **18:** 313 (1901).

[7] Anderson, C. G., "An Introduction to Bacteriological Chemistry," The Williams & Wilkins Company, Baltimore, 1938.

[8] Lucas, G. H. W., *Jour. Phys. Chem.,* **25:** 1180 (1921).

by the use of an alcoholic solution of barium hydroxide. The barium salt of one fraction, bios I, was insoluble in alcohol, while no salt was formed by the second fraction, which was designated as bios II. Neither of the fractions by itself had much activity, but a combination of the two fractions produced growth stimulation.

1. *Bios I.   i-Inositol.*—Eastcott[1] identified bios I as optically inactive inositol, which has the following structural formula:

$$
\begin{array}{c}
\text{CHOH} \\
\diagup \quad \diagdown \\
\text{HOHC} \qquad \text{CHOH} \\
| \qquad\qquad | \\
\text{HOHC} \qquad \text{CHOH} \\
\diagdown \quad \diagup \\
\text{CHOH} \\
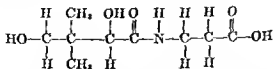\textit{i-Inositol}
\end{array}
$$

Inositol had very little action when used alone but was effective when combined with other components of bios.

2. *Bios II.*—Bios II, the portion of bios not precipitated by an alcoholic solution of barium hydroxide, has been fractionated into other substances by different methods.

a PANTOTHENIC ACID.—Using fuller's earth, Williams and his coworkers[2] separated bios II into an adsorbed fraction, replaceable by vitamin B_1 (thiamin or aneurin), and an unadsorbed fraction designated by them as "pantothenic acid."

The term "pantothenic" is derived from Greek and signifies "from everywhere." Pantothenic acid is thus named because it may be isolated from a large number of sources, which include plant (bacteria, slime molds, etc.) and animal tissues.

Pantothenic acid has been synthesized by Stiller, Keresztesy, and Finklestein[3] of the Merck Research Laboratories. It has the following chemical structure:

$$
\begin{array}{c}
\quad\ \text{H CH}_3\ \text{OHO}\quad\ \text{H H H O} \\
\qquad |\quad |\qquad |\ \ |\quad\ \ |\ \ |\ \ |\ \ | \\
\text{HO}-\text{C}-\text{C}-\text{C}-\text{C}-\text{N}-\text{C}-\text{C}-\text{C}-\text{OH} \\
\qquad |\quad |\qquad |\qquad\quad\ |\ \ |\ \ | \\
\quad\ \text{H CH}_3\ \text{H}\qquad\quad\ \text{H H H}
\end{array}
$$

It is prepared by condensing alpha-hydroxy-beta, beta-dimethyl-gamma-butyrolactone, a product that has been synthesized, with beta-alanine.

Small amounts of thiamin, or relatively large quantities of *i*-inositol,

[1] EASTCOTT, E. V., *Jour. Phys. Chem* , **32**: 1094 (1928).

[2] WILLIAMS, R. J., and E. BRADWAY, *Jour. Am Chem. Soc.*, **53**: 783 (1931); and HONN, *Jour. Plant Physiol.*, **7**: 629 (1932).

[3] WILLIAMS, R. J., and R. T. MAJOR, *Science*, **91**: 246 (1940).

or mixtures of the two, increased the stimulating effect of this acid in respect to certain yeasts [1]

b. BIOTIN.—If bios is fractionated with charcoal, the adsorbed portion contains a substance designated by Kögl and his associates [2] as "biotin." This substance may be eluted from charcoal by the aid of an aqueous solution of ammonia and acetone. Afterward the product may be purified. Kögl [3] states that approximately 360 tons of ordinary yeast would be necessary for the production of 1 g. of biotin. The unadsorbed portion has been called bios III

Biotin has been assigned the tentative formula $C_{11}H_{18}O_3N_2S$ It has been obtained as the methyl ester in crystalline form One part of biotin in 4×10^{10} parts of medium caused a distinct stimulation, while one part in 10^{11} parts produced a perceptible effect on the growth of yeast (strain M). The growth effect is increased by the presence of the cofactors i-inositol and thiamin.

c. BETA-ALANINE —Miller and his associates [4] have separated bios II into two fractions by the use of charcoal. The portion adsorbed by the charcoal and removed by shaking with an aqueous solution of acetone and ammonia was designated as "bios IIB," while the unadsorbed portion of bios II, the filtrate, was called "bios IIA" The properties of bios IIA are due to beta-alanine and leucine [5]

In a medium containing sugar, salts, and 5 mg of inositol per liter, the growth of several strains of yeasts was stimulated by the addition of as little as 0 08 microgram of β-alanine per cubic centimeter of medium (approximately 1 part in 12,000,000) Aspartic acid enhanced the effect. [6] Pantothenic acid, and thiamin in at least one case, stimulated growth when added

At very high dilutions, β-alanine may be slightly inhibitory [7] Nielsen and Hartelius state that the β-alanine is toxic to yeast except when asparagine, or aspartic acid, is a constituent of the medium [8]

d. THIAMIN.—Williams and his associates (1930) observed that thiamin (vitamin B₁, or aneurin) stimulated the growth of a certain strain of yeast. [9]

[1] WILLIAMS, R J., and D H SAUNDERS, *Biochem Jour*, 28: 1887 (1931)
[2] KÖGL, F., und B. TÖNNIS, *Zeit physiol Chem*, 242: 43 (1936) *Chem & Ind*, 57: 49 (1938)
[3] *Ibid*
[4] MILLER, W L., E V EASTCOTT, and L M SPARLING, *Trans Roy Soc Can* III, 26: 165 (1932)
[5] MILLER, W L., *Trans Roy Soc Can*, III, 30: 99 (1936)
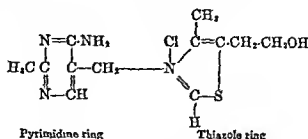[6] WILLIAMS, R J., and E ROHRMAN, *Jour. Am Chem Soc*, 58: 695 (1936)
[7] *Ibid*
[8] NIELSEN, N., und V HARTELIUS, *Biochem Zeit*, 296: 359 (1938)
[9] WILLIAMS, R J and R R RORNY, *Jour Biol Chem*, 87: 581 (1930)

Thiamin has the following structural formula:

$$
\begin{array}{c}
\text{CH}_2 \\
\text{N=C-NH}_2 \qquad\qquad \text{Cl} \quad \text{C=C-CH}_2\text{-CH}_2\text{OH} \\
\text{H}_3\text{C C} \quad \text{C--CH}_2\text{---} \text{N} \\
\text{N---CH} \qquad\qquad\qquad \text{C--S} \\
\text{H}
\end{array}
$$

Pyrimidine ring   Thiazole ring

Farrell[1] found that *Saccharomyces hanseniospora valbyensis* reproduced well in a medium containing tomato juice but not in a medium containing inositol, bios IIA, and bios IIB. She designated the substance that stimulated growth as "bios V". It has been shown that bios V may be replaced by thiamin.

Thiamin has a marked effect on yeast fermentation, according to Schultz and his coworkers.[3]

Thiamin, derivatives of thiamin, and the pyrimidine and/or the thiazole portions of the thiamin molecule may serve as accessory growth factors for certain microorganisms.[4]

Rhodotorula rubra and *R. flava*[5] are stimulated not only by the intact thiamin molecule but also by the pyrimidine ring of the molecule. A concentration of approximately 0.4 microgram of thiamin in 25 cc. of medium produced maximum growth.

The action of thiamin, pyrimidine, and thiazole on various yeasts has been investigated by Schultz and his associates.[6]

e. BIOS VII.—A "useful constituent," which accompanies bios IIB, has been named "bios VII" by Miller.[2] Marchant (1942) has shown that bios VII may be almost replaced by pyridoxine.

Sources of Bios Constituents.—Constituents of bios may be isolated from a large number of sources: from wild yeasts, from *Aspergillus niger*,[7] from the leaves and buds of the birch,[8] from malt rootlets,[9] from beer wort, from tomato juice, from the charcoal used in the refining of raw brown sugar, and from many other sources.

[1] FARRELL, L. N., *Trans Roy. Soc Can.*, III, 29: 167 (1935).

[2] MILLER, W. L., *Trans Roy Soc. Can*, III, 31: 159 (1937).

[3] SCHULTZ, A. S., L ATKIN, and C N FREY, *Jour. Am. Chem. Soc*, 59: 948 (1937).

[4] KOSER, S A., and F SAUNDERS, *Bact Rev*, 2: 99 (1938).

[5] SCHOPFER, W. H., *Compt. rend*, 205: 445 (1937), *Compt. rend. soc. biol*, 126: 812 (1937).

[6] SCHULTZ, A S., L ATKIN, and C. N. FREY, *Jour Am. Chem. Soc*, 60: 490 (1938)

[7] NIELSEN, N., und V. HARTELIUS, *Compt. rend. trav. lab. Carlsberg, Sér. physiol*, 22: 1 (1937)

[8] DAGYS, J, *Protoplasma*, 24: 14 (1935).

[9] DEAS, J., *Jour Biol. Chem.*, 61: 5 (1924).

Function of Growth Substances.—Thiamin is closely related to cocarboxylase and is believed to function in the structure of the latter compound.

The exact functions of biotin, inositol, β-alanine, and pantothenic acid are not known at present.

For further information concerning this subject, the reader is referred to the publications listed in the following bibliography, some of which contain additional references.

References Concerning the Growth Substances of Yeast

BUCHANAN, R E, and E. I. FULMER: "Physiology and Biochemistry of Bacteria," Vol. II, Williams & Wilkins, Baltimore, 1930

COPPING, A, M : The Effect of "Bios" on the Growth and Metabolism of Certain Yeasts, *Biochem Jour*, 23: 1050 (1929)

DAGYS, J.: Die Hefewuchsstoffe in Knospen und Blättern, *Protoplasma* 26· 20 (1936)

DEVLOO, R · Purification du bios de Wildiers, *Cellule*, 23: 361 (1906)

EASTCOTT, E. V : Wildiers' Bios   The isolation and identification of "Bios I," *Jour Phys. Chem*, 32: 1091 (1928)

EULER, H VON, and T PHILIPSON Wasserlösliche Wachstumsfaktoren, *Biochem Zeit*, 245: 418 (1932)

FARRELL, L. N.: The Influence of Inositol, Bios IIA and Bios IIB on the Reproduction of Twelve Species of Yeast   A New Constituent of Bios   *Trans. Roy Soc Can.*, III, 29: 167 (1935)

FULMER, E. I, W W DUECKER, and V E NELSON The Multiple Nature of Bios, *Jour Am. Chem. Soc*, 46: 723 (1921)

HALL, D. H , L. H JEANES, and S STUART: Yeast Growth Stimulants in White Sugars, *Ind Eng Chem*, 25: 1052 (1933)

KÖGL, F : On Plant Growth Hormones, *Chem and Ind*, 57: 49 (1938)

——— und B TÖNNIS: Über das Bios-Problem   Darstellung von krystallisierten Biotin aus Eigelb *Zeit physiol Chem*, 242: 43 (1936)

KOSER, S A, and F SAUNDERS: Accessory Growth Factors for Bacteria and Related Microorganisms, *Bact. Rev*, 2: 99–160 (1938)

LUCAS, G H. W.: The Fractionation of Bios and Comparison of Bios with Vitamins B and C, *Jour, Phys. Chem*, 28: 1180 (1921).

LUTMAN, B F.: "Microbiology," McGraw-Hill Book Company, Inc, New York, 1929

MARCHANT, C.: The Identification of Bios V as Vitamin B₁ and of a Constituent of Bios VII Solution as Vitamin B₆, Their Effect upon the Reduction of *Saccharomyces hanseniaspora valbyensis*, Yeast 2335, and *Saccharomyces galactosus*, *Can Jour Research*, 20: 21–31 (1942)

MILLER, W L : Wildiers' Bios, *Trans Roy Soc Can*, III, 29: 163 (1935)

———: Wildiers' Bios, *Trans Roy Soc Can*, III, 30: 99 (1936

———: Wildiers' Bios, *Trans Roy Soc Can*, III, 31: 159 (1937·

———, E V EASTCOTT, and E M STARLING· The fractionation of "Bios II," *Trans Roy Soc Can*, III, 26: 165 (1932)

NIELSEN, N, and V HARTELIUS The Separation of Growth Promoting Substances *Compt rend trav lab Carlsberg, Sér phynol*, 19: (No 8), 1932

—— und ——— Über die Trennung der auf die Stoffproduktion der Hefe und Schimmelpilzen einwirkenden Wuchsstoffe, *Compt rend trav lab Carlsberg, Sér phynol* 22 (No 1), 1937

——— und ———: Wuchsstoffwirkung der Aminosauren, *Compt rend trav lab Carlsberg, Sér. physiol.*, 22 (No. 14): 249 (1938).

——— und ———: Untersuchungen über die Wuchsstoffwirkung von β-Alanin, β-Alanyl-Glycin, Asparaginsäure, Glycyl-Asparaginsäure und verwandte Stoffe auf Hefe, *Biochem. Zeit*, 296: 359 (1938).

RICHARDS, O W : The Stimulation of Yeast Growth by Thallium, a "Bios" Impurity of Asparagine, *Jour. Biol. Chem.*, 96: 405–418 (1932).

——— and M C TROUTMAN: Spectroscopic Analysis of the Mineral Content of Yeast Grown on Synthetic and Natural Media, *Jour. Bact.*, 39 (No. 6): 739–746 (1940).

SCHADE, A. L : Growth Substances in Yeast, *Communications on the Science and Practice of Brewing*, No. 4, December, 1938.

SCHOPFER, W H : L'aneurine et ses constituants, facteurs de croissance de Mucorinées (Parasitella, Absidia) et de quelques espèces de Rhodotorula, *Compt. rend. soc. biol.*, 126: 842 (1937)

———· L'action des constituants de l'aneurine sur les levures (Rhodotorula rubra et flava), *Compt. rend Acad , Sci* , 205: 445 (1937)

SCHOPFER, W H.: "Plants and Vitamins," Chronica Botanica Company, Waltham, Mass., 1943.

SCHULTZ, A S , L ATKIN, and C. N. FREY: Thiamine, Pyrimidine and Thiazole as Bios Factors, *Jour Am Chem Soc*, 60: 490 (1938).

SPERTI, G. S , J R LOOFBOUROW, and C M DWYER: Proliferation-promoting Factors from Ultra-violet Injured Cells, *Studies Inst. Divi Thomae*, 1: 163 (1937)

STANTIAL, H.· The Effect of Inositol, of Bios II, and of Both Together in the Culture Medium, on the Reproduction of Twelve Kinds of Yeast, *Trans. Roy. Soc Can* , III, 26: 163 (1932).

TANNER, F W : The "Bios" Question, *Chem Rev* 10: 397 (1925).

THIMANN, K. V : Growth Substances in Plants, *Ann Rev. Biochem* , 4: 545 (1935).

WILDIERS, E : Nouvelle substance indispensable au développement de la levure, *Cellule*, 18: 313 (1901)

WILLIAMS, R J : Growth-promoting Nutrilities for Yeasts, *Biol Revs* , 16: 49–80 (1941).

———, C M LYMAN, G H. GOODYEAR, J H. TRUESDAIL, and D HOLADAY: "Pantothenic Acid," a Growth Determinant of Universal Biological Occurrence, *Jour Am. Chem. Soc.*, 55: 2912 (1933).

——— and R R ROEHM. The Effect of Antineuritic Vitamin Preparations on the Growth of Yeasts, *Jour Biol. Chem* , 87: 581 (1930).

——— and E ROHRMAN, β-Alanine and "Bios," *Jour. Am. Chem. Soc.* 58: 695 (1936)

——— and D H. SAUNDERS: The Effects of Inositol, Crystalline Vitamin B_1 and "Pantothenic Acid" on the Growth of Different Strains of Yeast, *Biochem. Jour* , 28: 1887 (1934).

———, J. L. WILSON, and F H. VON DER AHE: The Control of "Bios" Testing and the Concentration of a Bios, *Jour. Am. Chem. Soc.*, 49: 227 (1927).

Some General References Concerned with Yeasts

BALTATU, E : Mycoderma als echte Saccharomycetes, *Zentr. Bakt. Parasitenk* , Abt II. 101: 196–225 (1939).

BAUMANN, C. A., and F. J STARE: Coenzymes, *Physiol. Rev.*, 19: 353 (1939).

BEDFORD, C. L : Genus *Hansenula* Sydow. Morphological and Physiological Studies, Doctor's Thesis, University of California, Berkeley (1941).

———: A Taxonomic Study of the Genus *Hansenula*, *Mycologia*, 34: 628–649 (1942)

Buchanan, E. D., and R. E. Buchanan: "Bacteriology," 4th ed., The Macmillan Company, New York, 1939.

Burk, D., and C. K. Horner: Nitrogen Assimilation by Yeast, a Consideration of Yeast Nutrition and Metabolism, *Wallerstein Labs. Communs* 2 (No. 6): 5–23 (1939)

Custers, M. Th. J.: Onderzoekingen over het gistgeslacht *Brettanomyces*, Thesis, *Tech Hoogeschool*, Delft (1940). Translated by H. J. Phaff and H. C. Douglas, University of California, Berkeley.

Dickens, F.: Yeast Fermentation, *Biochem. Jour*, 32: 1615, 1626, 1645 (1938)

Diddens, H. A., und J. Lodder: Die anaskosporogenen Hefen, Zweite Hälfte, N. V. Noord-Hollandsche Uitgevers Maatschappij, Amsterdam, 1942

Elvehjem, C. A.: The Rôle of Iron and Copper in the Growth and Metabolism of Yeast, *Jour Biol. Chem*, 90: 111–132 (1931)

Fischer, A. M.: Yeast and Factors Determining Its Vitamin Potency, *Brewers Digest*, 13; 37 (No. 10) (1938)

Fred, E. B., and S. A. Waksman: "Laboratory Manual of General Microbiology," McGraw-Hill Book Company, Inc., New York, 1928

Frey, Charles N.: History and Development of the Modern Yeast Industry, *Ind. Eng Chem*, 22: 1154 (1930)

——, G. W. Kirby, and A. Schultz: Yeast. Physiology, Manufacture and Uses, *Ind. Eng Chem*, 28: 879–884 (1936)

——, A. S. Schultz, and L. Atkin: Factors Affecting the Fermentation Rate of Yeast, p. 332, Abstracts of Communications, Third International Congress of Microbiology, New York, Sept 2–9, 1939

Glaubitz, M: "Atlas der Gärungsorganismen," Paul Parey, Berlin, 1932

Gottschalk, A: The Mechanism of Selective Fermentation of d-Fructose from Invert Sugar by Sauternes Yeast, *Biochem Jour*, 40 (No. 4): 621–626 (1946)

Graham, V. R., and E. G. Hastings: Studies on Film-forming Yeasts, *Can. Research*, 19: 251–256 (1941)

Guilliermond, A: "The Yeasts," translated and revised by F. W. Tanner, John Wiley & Sons, Inc., New York, 1920.

——: "Clef dichotomique pour la détermination des levures," Librairie Le François, Paris, 1928

——: "La sexualité, le cycle de développement, la phylogénie, et la classification des levures d'après les travaux récents," Masson et Cie, Paris, 1937

—— et R. Gautheret: Action des bleus de Nil et de crésyl sur les levures réduction et excrétion de ces colorants par les levures, *Compt. rend. Acad. Sci*, 206: 1818 (1938)

Henrici, A. T.: "Molds, Yeasts and Actinomycetes," John Wiley & Sons, Inc., New York, 1930

——: The Yeasts Genetics, Cytology, Variation, Classification and Identification, *Bact Revs*, 5: 97–179 (1941)

Hopkins, R. H., and R. H. Roberts. The Kinetics of Alcoholic Fermentation of Sugars by Brewer's Yeast I Effect of Concentrations of Yeast and Sugar *Biochem Jour*, 29: 919 (1935). II The Relative Rates of Fermentation of Glucose and Fructose, *Biochem. Jour*, 29: 931 (1935) III The Temperature Coefficients of the Rates of Fermentation of Glucose and Fructose, *Biochem Jour*, 29: 2486 (1935). IV Specificity The Rates of Fermentation of α- and β-Glucose, *Biochem Jour*, 30: 76 (1936)

Jörgensen, A: "Practical Management of Pure Yeast," 3d ed., revised by A Hansen, Charles Griffin & Company Ltd, London 1936

JOSLYN, M A. The Mineral Metabolism of Yeast, *Wallerstein Labs. Communs*, 4 (No. 11): 49–65 (1941).

KLUYVER, A J, and M. TH J. CUSTERS: The Suitability of Disaccharides as Respiration and Assimilation Substrates for Yeasts Which Do Not Ferment These Sugars, *Antonie van Leeuwenhoek*, 6: 121–162 (1939, 1940).

KÖVESSI, F. Investigations on the Development of Yeast Cells (*Saccharomyces sp*), Mechanical Equations Expressing the Development of Living Beings, and the Biophysical-Chemical Relationships, p. 336, Abstracts and Communications, Third International Congress of Microbiology, New York, Sept. 2–9, 1939.

KRUIS, K, a J ŠATAVA: "O vývoji a klíčení spór jakož i sexualitě kvasinek," V Praze, 1918

KUFFERATH, M H.: How to Identify Yeasts, II. Asporogenous Yeasts, *Rev. fermentations et inds aliment*, 1 (No 4): 161 (1946).

LANDIS, Q, and C N FREY: The Enzymes of Flour in Relation to Yeast Fermentation, *Cereal Chem*, 13: 281 (1936).

LANGERON, M, and P GUERRA: Nouvelles recherches de zymologie, médicale, *Ann parasitol humaine et comparée*, 16: 36–81, 162–179, 429–476, 481–525 (1938).

LAUFER, S, and R. SCHWARZ: "Yeast Fermentation and Pure Culture Systems," Schwarz Laboratories, Inc., New York, 1936

LINDEGREN, C. C., and G LINDEGREN: Sporulation in *Saccharomyces cerevisiae*, *Botan Gaz* 105 (No 3): 304–316 (1944).

LODDER, J · Die anaskosporogenen Hefen, Erste Hälfte, *Verhandel. Konink. Akad Wetenschap Amsterdam, Afdeel. Natuurk.*, Sec. II, 32: 1–256 (1934).

LUTMAN, B F.: "Microbiology," McGraw-Hill Book Company, Inc., New York, 1929

MACFARLANE, M G : Phosphorylation in Living Yeast, *Biochem Jour.*, 30: 1369 (1936)

MCANALLY, R A, and J SMEDLEY-MACLEAN: The Synthesis of Reserve Carbohydrate by Yeast I Synthesis from Glucose and Maltose and the Influence of Phosphate Thereon, *Biochem Jour*, 29: 1872 (1935).

MCKATER, J. A . Cytology of *Saccharomyces cerevisiae* with especial reference to nuclear division, *Biol Bull Marine Biol Lab.*, 49: 213 (1927).

MRAK, E M, and S MCCLUNG: Yeasts Occurring on Grapes and in Grape Products in California, *Jour Bact.*, 40: 395–407 (1940).

———, H J PFAFF, and H. C DOUGLAS: A Sporulation Stock Medium for Yeasts and Other Fungi, *Science*, 96 (No. 2497): 432 (1942)

———, and R H VAUGHN: Yeasts Occurring on Dates, *Jour. Bact*, 43 (No. 6): 689–700 (1942)

———, ———, and H N HANSEN: Yeasts Occurring in Souring Figs, *Jour Bact*, 44 (No 4): 441–450 (1942)

NEUBERG, C · The Biochemistry of Yeasts, *Ann Rev Biochem.*, 15: 435–474, 685–687 (1946).

NICKERSON, W J : *Zygosaccharomyces Acidifaciens*: A New Acetifying Yeast, *Mycologia*, 36 (No 1) 66–78 (1943).

———. Some Trends in Research on Yeasts, *Chronica Botan.*, 7 (No. 8): 400–412 (1943)

———: Studies in the Genus *Zygosaccharomyces* I Transfer of Pellicle-forming yeasts to *Zygopichia*, *Farlowia*, 1 (No 3): 469–481 (1944)

———: Studies on Film-forming Yeasts Acid Production by *Zygopichia* and *Zygohansenula*, *Mycologia*, 36 (No. 3): 224–233 (1944)

——— and W R. CARROLL: On the Metabolism of *Zygosaccharomyces*, *Arch Biochem*, 7 (No 1): 257–271 (1945).

PETT, L. B.: Changes in the Flavine Content of Yeast. *Arkiv Kemi Mineral. Geol.*, 11B: No. 53 (1935).

——: Studies in Yeast Grown in Cyanide, II, *Biochem. Jour.*, 30: 1438 (1936).

PYLER, E. J., and T. P. KRUZIC: Yeast Propagation in the Modern Brewery, *Brewers Digest*, 14 (No. 2): 25–7T (1939).

RICHARDS, O. W.: Increased Growth of a Population of Yeast Obtained with Inosite, *Proc. Soc. Expt. Biol. Med.*, 29: 627 (1932).

——: The Effect of Deuterium on the Growth of Yeast, *Jour. Bact.*, 28: 289 (1934)

——: Colchicine Stimulation of Yeast Growth Fails to Reveal Mitosis, *Jour. Bact.*, 36: 187 (1938)

—— and A. J. KAVANAGH: The Course of Population Growth and the Size of Seeding, *Growth*, p. 217, September, 1937

ROBISON, R.: Chemistry and Metabolism of Compounds of Phosphorus, *Ann. Rev. Biochem.*, 5 (1936)

SALLE, A. J. "Fundamental Principles of Bacteriology," McGraw-Hill Book Company, Inc., New York, 1948

ŠATAVA, JAN: "O redukovaných formách kvasinek," V Praze, 1918

——: "Les formes sexuelles et asexuelles des levures et leur pouvoir fermentatif," IIIe Congrès international technique et chimique des industries agricoles, Paris, 1934

SCHADE, A. L.: Growth Substances in Yeast, *Wallerstein Labs. Communs.*, 2 (No. 4): 36–40 (1938)

——: The Yeasts, *Wallerstein Labs. Communs.*, 4 (No. 13): 192–193 (1941).

SCHOEN, M.: "The Problem of Fermentation," Chapman Hall, Ltd., London, 1928

—— Faits nouveaux et nouvelles hypothèses dans la chimie des fermentations (A review), *Ann. fermentations*, 4: 193–219, 257–283, 321–345 (1938)

SCHULTZ, A. S., and L. ATKIN: The Utility of Bios Response in Yeast Classification and Nomenclature, *Arch. Biochem.*, 14 (No. 3): 36 (1947)

SIZER, I. W.: Sucrose Inversion by Bakers' Yeast as a Function of Temperature, *Jour. Gen. Physiol.*, 21: 695 (1938)

SKINNER, C. E.: The Yeast-like Fungi. *Candida* and *Brettanomyces*, *Bact. Revs.*, 11 (No. 4): 227–271 (1947). (Article contains 303 references.)

—— and R. BOUTHILET: Melibiose Broth for Classifying Yeasts, *Jour. Bact.*, 53 (No. 1): 37 (1947).

SMYTHE, C. V.: Phosphoric acid esters from yeast extract, *Jour. Biol. Chem.*, 117: 135 (1937)

——: The utilization of pyruvic acid by bakers' yeast, *Jour. Biol. Chem.*, 125: 635 (1938)

—— The effect of certain tissue extracts, of ammonium salts and of certain amides on the rate of fermentation by bakers' yeast, *Enzymologia*, 6: 9 (1939)

STANTIAL, H.: The Sporulation of Yeast, *Trans. Roy. Soc. Can.*, III, 29: 175–188 (1935)

STELLING-DEKKER, N. M.: Die Sporogenen Hefen, *Verhandel. Koninkl. Akad. Wetenschap. Amsterdam, Afdeel. Natuurk.*, Sec. II, 28: 1–547 (1931)

STEPHANT, C. D., and H. W. VON LOESECKE: "Selected References on Yeast," War Food Administration, Office of Marketing Services, Washington, D. C., January 1945

TANNER, F. W., E. D. DEVEREUX, and F. M. HIGGINS: The Multiplication of Yeasts and Yeast-like Fungi Synthetic Nutrient Solutions, *Jour. Bact.*, 11: 45 (1926)

—— and L. MILLHOUSE: Observations on the growth of yeasts in pure nutrient solutions, *Proc. Soc. Expt. Biol. Med.*, 23: 447 (1926)

———— and L. B STRAUCH: Effect of sodium benzoate upon certain yeasts, *Proc. Soc. Expt. Biol. Med.,* 23: 449 (1926).

———— and B. W. WILLIAMSON: The Effect of Freezing on Yeasts. *Proc Soc. Expt Biol Med.,* 26: 377 (1928).

TAUBER, H.: "Enzyme Chemistry," John Wiley & Sons, Inc., New York, 1937.

THORNE, R S W.: The Nitrogen Nutrition of Yeast, *Wallerstein Labs. Communs,* 9 (No. 27) 97–114 (1946).

WALLERSTEIN, J. S.: New Facts About Growth Substances, *Wallerstein Labs. Communs,* 4 (No 11): 14–19 (1941).

————and A. L SCHADE: Some Considerations on the Nature of Yeast I. The Structure and Functions of the Cell, *Wallerstein Labs. Communs,* 3 (No. 9): 91–106 (1940)   II. Their Origin and Relation to Other Organisms, *Wallerstein Labs. Communs,* 3 (No. 10): 182–193 (1940).

WICKERHAM, L J.: A Critical Evaluation of the Nitrogen Assimilaton Tests Commonly Used in the Classification of Yeasts, *Jour Bact,* 52 (No. 3): 293–301 (1946).

————: A Simple Technique for the Detection of Melibiose-fermenting Yeast, *Jour. Bact,* 46 (No. 6): 501–505 (1943)

———— and A A ANDREASEN: The Lyophil Process   Its Use in the Preservation of Yeast, *Wallerstein Labs. Communs,* 5 (No. 16): 165–168 (1942).

———— and E DUPRAT: A Remarkable Fission Yeast, *Schizosaccharomyces versatilis* Nov Sp, *Jour. Bact,* 50 (No. 5)· 597–607 (1945).

———— and M. H. FLICKINGER: Viability of Yeasts Preserved Two Years by The Lyophil Process, *Brewers Digest,* 21 (No. 4) (April, 1946).

————, ———— and K.A. BURTON: A Modification of Henrici's Vegetable-juice Sporulation Medium for Yeasts, *Jour. Bact.,* 52 (No 5): 611–612 (1946).

———— L B LOCKWOOD, O. G. PETTIJOHN, and G. E. WARD: Starch Hydrolysis and Fermentation by the Yeast *Endomycopsis fibuliger, Jour Bact.,* 48 (No. 4): 413–427 (1944)

WINGE, Ö : On haplophase and diplophase in some Saccharomycetes, *Compt. rend. trav lab Carlsberg, Sér physiol,* 21 (No. 4): 77 (1935)

————and O. LAUSTSEN: On two types of spore germination, and on genetic segregations in Saccharomycetes, demonstrated through single-spore cultures. *Compt rend trav lab Carlsberg, Sér. physiol,* 22 (No. 6): 99 (1937).

———— and ————: Artificial species hybridization in yeast, *Compt. rend. trav. lab Carlsberg, Sér. physiol,* 22 (No. 13): 235 (1938)

Some References Concerning Yeast Genetics

GARLAN, P.: Fundamentals of Genetics, *Wallerstein Labs. Communs.,* 9 (No. 26): 5–27 (1946)

HENRICI, A. T.: The Yeasts, Genetics, Cytology, Variation, Classification and Identification, *Bact. Revs.,* 5: 97–179 (1941)

KRUIS, K., and J. ŠATAVA: O vývoji a kličení spór jakož; sexua litě kvasinek, V Praze (1918)

LINDEGREN, C C.: The Improvement of tion, *Wallerstein Labs. Communs.,*   . . .   . hybridiza-thesis,

————: Yeast Genetics, Life Cycles, · · ·
and Adaptive Enzymes, *Bact. Revs.,* 9: 111–170 (1946).

————: Breeding Yeasts for Their New Role in Nutrition, *Missouri Botan. Garden Bull ,* 34: 37–43 (1946).

———— and G. LINDEGREN: Vitamin-synthesizing Deficiencies in Yeasts Supplied by Hybridization, *Science,* 102 (No. 2637): 33–34 (1945).

————, S. Spiegelman, and G. Lindegren, Mendelian Inheritance of Adaptive Enzymes in Yeast, *Proc. Nat. Acad. Sci*, **30**: 316–352 (1944).

Šatava, J.: Oredukovanŝch formâch krasnek V. Praze (1918)

————: Les formes sexuelles et asexuelles des levures et leur pouvoir fermentatif—IIIe Congrès international technique et chimique des industries agricoles, Paris (1918).

Spiegelman, S, and C. C. Lindegren: The Relation of Sporulation and the Range of Variation of the Haplophase to Population Adaptation, *Jour. Bact*, **49**: 257–264 (1945).

————, ———— and G. Lindegren. Maintenance and Increase of a Genetic Character by a Substrate–Cytoplasmic Interaction in the Absence of the Specific Gene, *Proc. Nat. Acad. Sci*, **31**: 95–102 (1945).

Winge, Ö.: On Haplophase and Diplophase in Some Saccharomycetes, *Compt. rend. trav. lab Carlsberg, Sér. physiol.*, **21** (No 4). 77–111 (1935)

———— and O Laustsen: On Two Types of Spore Germination, and on Genetic Segregations in *Saccharomyces*, Demonstrated through Single-spore Cultures, *Compt. rend. trav. lab. Carlsberg, Sér. physiol.*), **22** (No. 6)· 99–116 (1937)

———— and ————: Artificial Species Hybridization in Yeast, *Compt. rend. trav. lab. Carlsberg, Sér. physiol.*, **22** (No. 13). 235–244 (1938)

———— and ————: On 14 New Yeast Types, Produced by Hybridization, *Compt. rend. trav. lab. Carlsberg, Sér. physiol.*, **22**, 337–352 (1939).

———— and ————: *Saccharomycodes ludwigii* Hansen, A Balanced Heterozygote, *Compt. rend. trav. lab. Carlsberg, Sér. physiol.*, **22**: 357–370 (1939)

———— and ————: On a Cytoplasmic Effect of Inbreeding in Homozygous Yeast, *Compt. rend. trav. lab. Carlsberg, Sér. physiol.*, **23**: 17–39 (1940)

CHAPTER III

SACCHARIFYING AGENTS: METHODS OF PRODUCTION AND USES

Introduction.—Starches, hemicelluloses, and celluloses must be hydrolyzed or converted to fermentable sugars by enzymatic or chemical agencies before they may be used for certain industrial purposes, such as ethanol production. A variety of methods may be used for converting complex carbohydrates to relatively simple materials. Some of these methods are applicable in the case of starches alone and others, with suitable modifications, may be used for the saccharification of either starches or cellulose materials.

Methods of Saccharifying Starch-containing Materials.—In general, the methods of saccharifying starch-containing materials involve the use of enzyme preparations or dilute acids, or a combination of the two. The enzyme preparations that may be used include malt, which is of cereal origin, and those of microbial origin, among which are mold and bacterial products. Illustrative of preparations of fungal origin are mold bran and fungal amylases. Either liquid or solid bacterial preparations are available. A method that depends upon growing an amylase-producing mold in the substrate that is to be fermented after saccharification is known as the Amylo process. Dilute acids, particularly hydrochloric acid, may be used to convert grains, potatoes, and other starch-containing raw materials. Various combinations of malt with mold bran, or other enzyme preparations of microbial origin, may be employed; or combinations of acid with mold enzymes may be used. The method of conversion selected will depend, of course, on the use intended and the availability of the hydrolytic agents and their relative costs.

Cooking the Mash.—The purpose of cooking grain mashes is to prepare them for the conversion process in which starches are saccharified to fermentable sugars. During the cooking, the starches are solubilized and gelatinized.

Cooking may be accomplished by batch or continuous processes. In batch operations, cooking may be done at atmospheric pressure with temperatures below the boiling point of water by methods known as infusion processes; or under pressure. The continuous processes utilize high temperatures, obtained by the use of high steam pressures, and short periods of time, usually less than one minute.

Infusion Process.—Some materials, such as wheat, rye, rye malt, and barley malt, may be adequately cooked by infusion processes. For example, wheat may be prepared in accordance with the following method of Stark, Adams, Scalf, and Kolachov.[1] Ground wheat is added slowly to water at a temperature of 100°F, while being agitated continuously. The temperature of the mash is elevated to 155°F. durng a period of 45 min., held at 155°F. for 60 min, and then lowered to 152°F. At this temperature a slurry of malt may be added Conversion may be carried out at 145°F. for 30 min.

Batch-type Pressure Cooking.—This process is carried out in tanks[2] of large capacity, for example, 10,000 gal or more The tanks are equipped with agitators, inlets for the water and the grain, outlets, steam spargers, usually a means for producing vacuum, and with other accessories

During operation, water at a temperature of 120 to 140°F. is added to the tank in an amount sufficient to supply 18 to 22 gal for each bushel of corn or other grain. Sulphuric acid is added to bring the pH of the mash to 5 4 to 5.6. Then the ground corn is added slowly, with agitation The mash is heated to 305 to 310°F by steam under pressure during a period of 45 min. and held for 5 to 10 min. It is cooled to 152°F, first by blowing it down to atmospheric pressure, and second by using vacuum

The comparatively large floor space occupied by the tank, the relatively long period of processing (3 to 4 hr), and the difficulty of obtaining uniform mixing and temperature are disadvantages

Continuous Pressure Cooking.—The most modern method for cooking corn and other grains is a continuous pressure process, which was developed by Unger.[3]

The effects of variables on the efficiency of operation was studied by Unger. The variables were (1) the temperature of cooking, (2) the time of cooking, (3) the particle size of the ground grain, (4) the ratio of the amount of grain to water, (5) the pH of the mash, and (6) agitation He found that the most important variables were temperature and time and that corn meal may be adequately cooked in 16 to 60 sec at 350 to 365°F, in a Schutte-Koerting jet heater He showed that grinding was not too important a factor, provided that the grain was ground enough to be cooked properly The optimum practical pH range was 5 4 to 5 6

A continuous cooker system with a capacity for 5,000 bu of grain per day was designed by Unger and constructed (refer to Fig 8)

[1] Stark, W H, S L Adams, R E Scalf, and P Kolachov, *Ind Eng Chem*, 35: 443 (1913)
[2] Unger, E D, Thesis, Case School of Applied Science, May, 1941
[3] *Ibid*
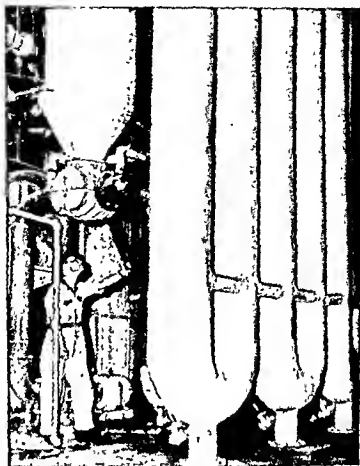
Fig. 8.—Tubes for continuous cooking. The mash is heated instantaneously with steam to 350 to 360°F by a jet heater. It passes through a series of tubes where it is held for 60 to 70 sec. It is then cooled to 145°F in a vacuum flash chamber. (*Courtesy of Joseph E. Seagram & Sons, Inc., Louisville, Ky.*)

For further details, the reader is referred to other sections of this book, to "Food for Thought" by Willkie and Kolachov, to Unger's thesis, and to other sources.

MALT

Malt is an enzymic product prepared usually from selected barley, although other cereal grains may be used.

The Preparation of Malt.—The manufacture of malt, commonly carried out by malsters, consists of steeping selected, screened barley, permitting it to germinate, and drying it under carefully regulated conditions. A schematic diagram of a malting plant is shown in Fig. 9.

Selection of Barley.—Barley is selected for the results that it produces. It should be of a good variety, preferably large grained, of a fairly uniform size, and of a light yellow color when matured. It should possess high germinating power, contain but a small amount of bitter and harsh substances in the husk, and have the ability to produce enzymes of the

proper character and quantity during germination. Soft, starchy barley is desirable; damaged or weedy barley is undesirable

Certain types of barley are sought for the manufacture of fine beer. In the United States malt is prepared principally from six-rowed barley, although some two-rowed barley is used in California and much of it in Europe.

Composition of Barley.—Barley contains the four plant proteins: glutelin, hordein, leucosin, and edestin. Glutelin and hordein are found

GRADERS AND CLEANERS
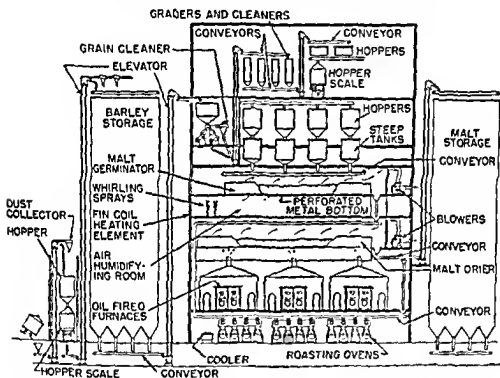
Fig. 9.—Malting plant (schematic diagram not drawn to scale)   *(Courtesy of the Editor, Food News, October, 1945.)*

mainly in the husk and aleurone cell layers; leucosin and edestin in the endosperm between the starch granules

Starch is found in the endosperm and is a most important constituent. Fat, rich in lecithin, is found in the embryo and in the aleurone cells. Tannins and bitter resins are located principally in the husks Together with the protein, the tannins and bitter resins are collectively referred to as "testinic acid."

Storing, Cleaning, Grading.—After receipt at the malting plant, the barley is weighed, stored, cleaned, and graded. It may be stored in bins, according to the variety From the bins, it is conveyed to the cleaners which are of various designs and construction but which generally employ

aspirators for blowing out light material, such as chaff, and screens for separating out stones, broken barley, and other extraneous material. The barley is then graded for size, each different grade going to a different holding tank or hopper and being processed separately.

Steeping.—Steeping is a process of soaking grain in order that it may take up sufficient moisture to quicken the living cells of the embryo and start the processes of enzyme production and germination, which precede the breakdown of cell walls and the hydrolysis of the stored foods

Before the barley is steeped it should be screened or sorted in order to obtain grains of a uniform size. The moisture content can then be controlled more readily during steeping.

During steeping, water is imbibed by the individual grains until an optimum moisture content of 45 to 47 per cent is obtained. This concentration of water favors normal germination.

Water of a known chemical composition and of a definite pH should be used during the steeping process. The composition of the water has much to do with the proper removal of the tannin, bitter resin, and some proteins, which are undesirable for beers. Since these substances dissolve more readily at a higher pH, the steeping water is sometimes made alkaline by the use of lime. The use of alkaline steep water apparently is of advantage when malt, and subsequently beer, is made from coarse barleys, but of no advantage when fine-skinned barleys are used.

Hypochlorites and permanganates have been used to some extent in the steep water It is claimed that they may stimulate germination and destroy harmful microorganisms in the water. The value of their use is not firmly established, however.

The rate at which the water is absorbed by the grains depends on the variety of the barley, the size of the grain and, mainly, the temperature of the water

A temperature of 50 to 60°F. in the steeping tanks is satisfactory. Control of the temperature is very important.

Malting is essentially a vital process involving growth and respiration. Respiration rates increase with a rise in temperature and with augmented water content, and oxygen is naturally required. Therefore aeration of the steep water is essential. Aeration also causes foreign material and small defective or light barley grains to rise to the surface of the steeping tank where they may be removed by skimming. As an alternate method of aeration, the water may be drained off and the tank refilled.

Understeeping results in a reduced rate of respiration, abnormal growth of the rootlets and an incomplete breakdown of the proteins. Less extract can be secured from the final product.

Oversteeping leads to the production of a higher percentage of ungerminated grains, caused by a deficiency in the oxygen supply during steeping, the inadequacy being due to the increased rate of respiration. If the water is adequately aerated, the grains will germinate subsequently, but the development is likely to be abnormal. Hence oversteeping may lead to low yields of malt.

Germination.—During germination several complex changes take place. The visible morphological changes include the formation of the acrospire, or plumule, and rootlets. A histological examination would show the disappearance of the cell walls of the endosperm, while a biochemical analysis would indicate that certain metabolic changes had taken place—the breakdown of proteins, starches, and other complex constituents under the influence of enzymes. Enzymes are elaborated or activated when the temperature, moisture, and aeration conditions are satisfactory for the germination of the seed.

The temperature, the moisture content of the grains, and the oxygen supply are very important during germination. These are controlled carefully in compartments or in revolving drums. Compartments are usually long and narrow. They are equipped with perforated metal bottoms, which allow temperature-controlled, humidified air to pass up through and water to drain out. Conveyors or spouts distribute the steeped barley over the compartment floor. During the germination process, the barley is aerated with humidified air and stirred by special screw propellers which constantly travel back and forth from one end of the compartment to the other, and may be watered by overhend devices. The temperature of the grain is maintained between 60 and 70°F by controlling the temperature of the incoming air. In rotating drums the humidified and temperature-controlled air enters each drum through a series of inlet tubes located near the periphery and leaves through an outlet near the center. The drums revolve slowly, usually at 1 to 2 r.p.m. during the germination process. Germination generally requires 5 to 7 days. The process is complete when the acrospire has grown to a length equal to three-fourths to the full length of the kernel.

The oxygen supply is especially important during germination, for the rate of respiration increases greatly in germinating grain. During respiration, heat energy is evolved, while carbon dioxide and water are produced as waste respiratory products. The accumulation of carbon dioxide inhibits normal respiration, and its concentration or a deficiency of oxygen, or both, induces abnormal respiration in which hydrogen acceptors other than oxygen may function. The products formed during abnormal respiration may include acids, alcohols, and aldehydes, which exert a toxic effect on the germinating grain or young plant.

Germinating barley usually shows a respiratory coefficient of approximately 1.

$$\text{Respiratory coefficient} = \frac{\text{volume } CO_2 \text{ evolved}}{\text{volume } O_2 \text{ absorbed}}$$

In the case of abnormal respiration, the respiratory coefficient increases, for the carbon dioxide increases, the oxygen decreases, or both changes occur simultaneously. For these reasons it is most important to secure good carbon dioxide removal and to turn and properly aerate germinating barley.

Drying the Germinated Barley.—When the barley has reached the desired stage of growth, drying stops the germination processes and with the application of heat develops color, flavor, and aroma and reduces the moisture content of the malt. A final moisture content of approximately 5 per cent is sought. Malt may then be stored without danger of breakdown by microorganisms.

Drying is carried out in thermostatically controlled kilns or drums at carefully regulated temperatures. The temperatures used have much to do with the enzyme content of the final malt and the flavor of beers made from it. Since much damage can be done to germinated barley by the application of too high temperatures when the grain is wet, low temperatures must be used at first. The temperature is raised gradually, or elevated by small increments, until a final kilning temperature of 75 to 100°C is used.

The final temperature used depends upon the nature of the malt desired. For a light malt, a lower temperature should be used; for a dark malt, a higher temperature.

During recent years, some malts have been dried in vacuum drums. Such malts possess a high concentration of enzymes but, though satisfactory for use in distilleries, are not so suitable for brewing, since the usual protein changes are affected.

Following the kilning, the malt may be treated, usually by friction, to remove the radicles.

Uses.—Malt has many uses. It is used extensively in the brewing industry as the chief raw material in the manufacture of beers and ales; and as the saccharifying agent in the manufacture of industrial alcohol and distilled liquors from grains, such as wheat, corn, and rye. Malt is also used in the manufacture of malted milk, candies, cereals, and food colorings but is usually roasted first in special ovens under carefully controlled conditions.

Rapid Conversion of Mash.—Gallagher and his associates[1] have described a fast method for converting grain mashes, which was based on

[1] GALLAGHER, F. H., H. R. BILFORD, W. H. STARK, and P. J. KOLACHOV, Ind. Eng. Chem., 34: 1395 (1942).

experimental data of the following nature secured in the laboratory. Corn cooks (mashes) were prepared under the same conditions and cooled to 62.8°C., the temperature of conversion. Barley malt (10 per cent), in the form of a slurry, was added in equal amounts to each cook. The mixture of malt and corn was agitated mechanically and conversion was carried out for specified times. The individual mashes were cooled quickly (within 7 to 10 min.) to 22.2°C. and made up to a concentration equivalent to 38 gal. of mash per bushel of grain. The pH was adjusted to 4.8 to 5.0. Then each mash was divided into four portions, three of which were inoculated with *S. cerevisiae* (Seagram No 1 strain) and one of which was reserved for control purposes. Fermentation was permitted to continue for 68 to 72 hr., after which the mashes were analyzed for residual sugar and alcohol. Data based on conversion periods of 1, 5, 15, 30, and 45 min. are presented in Table 10.

TABLE 10.—COMPARISON OF EFFECT OF MASH CONVERSION TIMES ON FERMENTATION YIELDS[1]

Conversion time, min	Total sugar			Efficiency, per cent		Grams absolute alcohol in 100 cc.
	Initial g /100 ml	Final g /100 ml	Fermented, per cent	Fermentation	Plant basis	
1	11 61	0 627	94 5	98 4	93 1	5 52
		0 629	94 5	99 1	93 8	5 56
		0 630	94 5	98 4	93 1	5 52
5	11 58	0 616	94 6	99 3	93 9	5 56
		0 672	94 2	99 4	93 8	5 55
		0 685	94 0	100 7	94 8	5 61
15	11.54	0 745	93 5	100 2	93 5	5 52
		0 715	93 8	101 1	94 7	5 59
		0 752	93 8	100 0	93 4	5 51
30	11.28	0 810	92 8	99 3	92 2	5 31
		0 856	92 4	102 5	94 8	5 46
		0 792	93 1	95 0	88 5	5 10
45	11.87	0 856	92 8	98 0	90 9	5 52
		0 885	92 5	89 1	82 3	5 00

[1] GALLAGHER, F. H., H R. BILFORD, W H STARK, and P J KOLACHOV, *Ind. Eng Chem.* **34:** 1395 (1942).

Additional data obtained by Gallagher, and his coworkers[1] showed that the 1-min. conversion period was equal to or better than the 60-min. period at a pH of 5 4 or 5 9, the lower and upper limits, respectively, of

[1] GALLAGHER, F. H., H. R. BILFORD, W. H STARK, and P. J KOLACHOV, *Ind. Eng Chem*, **34:** 1395 (1942).

levels optimum for plant conversions. More than 70 per cent of the cooked starch in the grain was converted to maltose by barley malt in 1 min. at 62.8°C.

The rapid method for the conversion of mashes is as follows: A proportioning pump continuously injects a slurry of malt into a stream of cooked mash which is being forced along by another pump. The products flow through a pipe 105 ft. long and 4 in. in diameter at a temperature of 62.8°C. The malt acts on the grain for a period of 40 sec. The mixture is then cooled during a period of 1.5 min. to 21.1 to 23.9°C. This particular unit has a capacity for converting 5,000 bu. of grain per day.

THE BALLS-TUCKER PROCESS

This is a process for utilizing the enzymes normally occurring in wheat for saccharification purposes in order to save malt.[1] Wheats contain adequate amounts of beta-amylase but are deficient in alpha-amylase. Accordingly, it is necessary to use some malt to compensate for the paucity in α-amylase.

The process is briefly as follows: A slurry of ground wheat is acidified to a pH of 5.2 to 5.8 and 0.05 to 0.1 per cent of Na_2SO_2 is added to activate the inactive form of amylase and to aid in the flocculation of wheat gluten. The mixture is agitated vigorously (for 1 hr. at 25°C. in one modification) and then permitted to stand for 45 min. or longer. The starch settles out and a considerable portion of the gluten rises to the surface and may be recovered. A portion of the clear supernatant fluid of the middle layer, which contains the activated amylases, is removed and later used to saccharify the cooked mash. Generally about 20 to 35 per cent of the wheat used may be extracted with sulphite. At least 2 per cent of malt on the basis of the weight of the grain is necessary for saccharification. For further details, the reader is referred to the report on "The Production of Ethyl Alcohol from Wheat" by the Northern Regional Research Laboratory, U S. Department of Agriculture, March, 1944

MOLD BRAN

Mold bran is an enzyme product obtained by growing *Aspergillus oryzae* on moist, sterilized bran. It is and may be used successfully as a substitute for malt in the saccharification of grain, potato, or other types of starch-containing mashes. Smaller quantities of mold bran than of malt are required to saccharify a given quantity of grain mash. Yields of ethyl alcohol from mashes saccharified with mold bran are, on the average, equal to and often superior to those obtained from mashes saccharified with malt

[1] BALLS, A. K., and I W. TUCKER, *Fruit Products Jour*, 23: 15–16, 21 (1943)

Development of Process.—Takamine,[1] in 1914, advocated the use of mold enzymes (from *A oryzae*) in the distilling industry. Studies were carried out in distilleries in Canada using his mold-bran preparation (*Taka-koji*) in place of malt to saccharify grains. Although the yields of alcohol obtained through the use of mold bran were reported to be higher than those obtained through the use of the malt, the process was not adopted.

In 1939, Underkofler, Fulmer, and Schoene[2] demonstrated by laboratory methods that higher yields of ethyl alcohol, on the average, could be obtained from corn mashes saccharified by the use of mold bran than from those saccharified by the use of barley malt. This work was later confirmed by them and their associates at the Iowa State College[3,4,5,6] and by Roberts, Laufer, Stewart, and Saletan.[7] Similar findings on a commercial basis have been reported by Boyer and Underkofler;[8] Underkofler, Severson, and Goering;[9] and Underkofler, Severson, Goering, and Christensen.[10] The favorable use of mold bran for the conversion of potato mashes was reported by Beresford and Christensen.[11]

Molds Used.—Hao, Fulmer, and Underkofler[12] studied mold bran prepared from 27 strains of molds of the genera *Aspergillus, Mucor, Penicillium,* and *Rhizopus.* They found that bran preparations made with strains of *A. oryzae, R. delemar,* and *R oryzae* were optimum for the saccharification of corn mashes as evaluated by the yields of ethanol produced. Strains of *A. oryzae* were selected as being best suited for industrial use, based on ease of handling, consistency of results, and high yields of alcohol from the saccharified mashes.

Table 11 shows the effect of saccharification temperature and time on

[1] TAKAMINE, J, *Ind. Eng. Chem.,* 6: 824 (1914)

[2] UNDERKOFLER, L A., E. I. FULMER, and L. SCHOENE, *Ind Eng Chem,* 31: 734 (1939)

[3] SCHOENE, L., E. I. FULMER, and L. A. UNDERKOFLER, *Ind Eng Chem,* 32: 544 (1940).

[4] UNDERKOFLER, L. A., *Brewers Digest,* 17 (No 11). 29 (1942)

[5] HAO, L. C., E. I. FULMER, and L. A. UNDERKOFLER, *Ind Eng Chem,* 35: 814 (1943)

[6] UNDERKOFLER, L. A., and E. I. FULMER, *Chronica Botan.,* 7: 120 (1943)

[7] ROBERTS, M., S. LAUFER, E. D. STEWART, and L. T. SALETAN, *Ind Eng Chem,* 36: 811 (1944).

[8] BOYER, J. W., and L. A. UNDERKOFLER, *Chem & Met Eng.* (December, 1915)

[9] UNDERKOFLER, L. A., G. M. SEVERSON, and K. J. GOERING, *Ind Eng Chem,* 38: 980 (1946).

[10] UNDERKOFLER, L. A., G M. SEVERSON, K J. GOERING, and H. M. CHRISTENSEN, *Cereal Chem,* 24 (No 1): 1 (1947)

[11] BERESFORD, H., and L. M. CHRISTENSEN, *Bull* 244, *Idaho Agr Expt Sta,* September, 1941.

[12] HAO, FULMER, and UNDERKOFLER, *loc cit*

alcohol yields from corn mashes saccharified by bran preparations made from selected strains of molds.

Production.—Mold bran may be produced by laboratory, pilot-plant, or commercial methods, some of which are described below.

Laboratory Methods.—The present discussion will be confined to a drum, a pot, and a tray method for preparing mold bran.

DRUM METHOD.—Spore cultures are prepared in flasks to serve as inoculums for the moist, sterilized bran in drums, in accordance with a procedure devised by Underkofler, Severson, Goering, and Christensen.[1]

TABLE 11.—EFFECT OF SACCHARIFICATION TEMPERATURE AND TIME ON ALCOHOL YIELDS FROM CORN MASHES SACCHARIFIED BY SEVERAL MOLD-BRAN PREPARATIONS[1]

Mold used in mold-bran preparation	Saccharification		Mold bran as per cent of corn	Alcohol yield as per cent of theoretical
	Temperature, °C.	Time, hr.		
Aspergillus oryzae (A.T.C.C No. 4814)*	30	1	4	88 6
	30	1	6	91.5
	30	1	8	91.3
	55	1	4	87 0
	55	1	6	88.4
	55	1	8	89 5
	55	2	4	86 1
	55	2	6	88.0
	55	2	8	89 5
Aspergillus oryzae (Rohm and Haas No 38)†	30	1	6	94 5
	30	1	8	94 8
	55	3	6	93 5
	55	3	8	93 7
Rhizopus oryzae (Lockwood No 660)‡	30	1	6	92 0
	30	1	8	93 5
	55	3	6	93 0
	55	3	8	94 0
Rhizopus delemar (NRRL No 1472)§	30	1	6	92 5
	55	3	6	91.0

[1] HAO, L C , E I FULMER, and L A UNDERKOFLER, *Ind Eng. Chem*, **35**. 814 (1943).
* American Type Culture Collection, Washington D. C.
† Rohm and Haas Co , Bristol, Pa.
‡ Dr. L. B Lockwood, U S Department of Agriculture
§ Northern Regional Research Laboratory, U.S Department of Agriculture, Peoria, Ill.

Ten-gram amounts of the following medium are distributed in 250-ml. flasks and sterilized in the autoclave: 10 g. of ground corn, 100 g of wheat bran, and 60 ml. of 0 2 N hydrochloric acid containing 0.62 p.p.m. of

[1] UNDERKOFLER, SEVERSON, GOERING, and CHRISTENSEN, *loc cit.*

$ZnSO_4 \cdot 7H_2O$, 0.63 p.p.m. of $FeSO_4 \cdot 7H_2O$, and 0 08 p.p m of $CuSO_4 \cdot 5H_2O$. After cooling, the moist, sterilized bran is inoculated with a culture of *A. oryzae.* The mixture is then distributed on one side of the flask and incubated at 30°C. After sporulation has reached a maximum, rapid drying of the culture is encouraged. The dry spore culture should be greenish in color, according to Underkofler and his associates, and not brown or black. The ratio of the inoculum for moist, sterile bran is about 1 per cent.

The well-sporulated bran culture thus prepared is used to inoculate a much larger charge (1,000 to 1,200 g.) of sterile bran of similar moisture content contained in a drum of 5-gal. capacity and capable of being rotated. During operation, air is passed slowly into the drum, which is maintained at a temperature of about 30°C. preferably (never above 35°C.) to favor the growth of the mold. During the germination period, the drum is rotated for 15 to 20 min or less each 2 hr. Thereafter, the drum may be rotated continuously at not more than 1 r p.m. until, at the end of 40 to 54 hr., the moldy bran is removed from the drum. It is then spread thinly on a papered surface and dried at room temperature. Sporulation proceeds during the drying. Before being used, the dried product is ground in the same manner as malt.

POT METHOD.—Hao, Fulmer, and Underkofler[1] developed a laboratory method for the production of mold bran in 1943 which they stated was superior in the following respects to the drum method used earlier: The equipment required less space and was less complicated; the mold mycelium was not disturbed during growth; and uniform aeration was obtained. Furthermore, the molds grew more rapidly in the pots and the resultant mold bran produced relatively consistent and higher yields of ethanol. The method consisted of growing selected strains of molds on wheat bran mash in 3-qt. aluminum pots especially equipped for aeration The apparatus, a modification of that used by Beresford and Christensen,[2] is shown in Fig. 10.

A detailed description of the pot method follows:

A bran mash is prepared by mixing 750 g of 0.3 N hydrochloric acid with 750 g. of wheat bran. The moistened bran is packed into the aluminum pot and sterilized with steam at 15 lb. pressure for 30 min The mash is cooled and then inoculated with 5 to 10 g of a well-sporulated culture of the mold (usually a strain of *A. oryzae*) which has been grown on wheat bran in flasks as described above. The seeded bran mash is packed firmly into the special pot, the latter being placed on a layer of cotton batting. The pot is incubated at 30°C. but the temperature rises

[1] Hao, Fulmer, and Underkofler, *loc. cit*
[2] Beresford and Christensen, *loc. cit.*

because of rapid growth of the mold and in about 8 hr. reaches 37 to 40°C. Air is passed through the mass at a pressure equal to 0.3 to 3 in. of water and at such a rate that the temperature is kept below 45°C. Aeration is continued for 12 to 24 hr. longer and then the moldy bran is removed from the pot, spread uniformly on paper, and permitted to dry at room temperature.
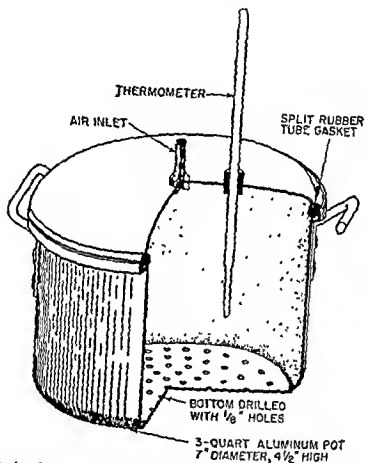
FIG. 10.—Apparatus for growing mold [*Courtesy of L. C Hao, E. I. Fulmer, and L. A. Underkofler, Ind Eng Chem., 35: 814 (1943)*]

TRAY METHOD.—The following method, described by Roberts and his collaborators,[1] is employed for preparing mold bran in trays Equal weights of wheat bran and water are mixed, and the mixture is spread in layers 1 in. deep in aluminum trays. The moistened bran is sterilized in the trays with steam at a pressure of 20 lb. for 30 min., cooled, and inoculated with a culture of *A. oryzae*, using aseptic technique. The seeded bran is incubated at 25°C. until the mold begins to sporulate, which generally requires about 4 days. The mold spores may be destroyed by adding 95 per cent ethanol to the mold-bran mixture in the proportion of

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[1] ROBERTS, LAUFER, STEWART, and SALETAN, *loc. cit*

1,500 ml. of ethanol to 500 g. of bran and 500 g. of water. The alcoholic extract is removed with a hydraulic press. The resulting dried, ground press cake is known as "Polidase-C."

Pilot-plant Methods.—These are concerned principally with problems of converting laboratory procedures to plant-scale operation and are based on the report by Underkofler, Severson, Goering, and Christensen [1]
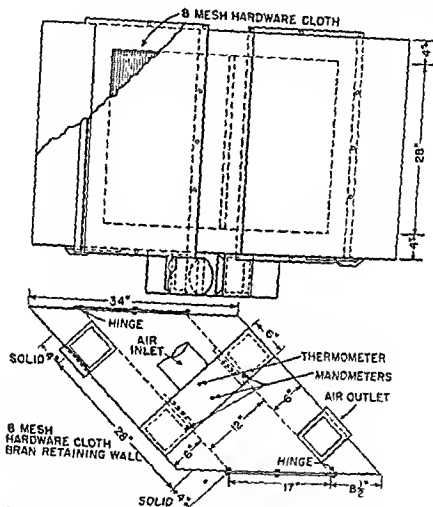

STEAM SUPPLY DETAIL
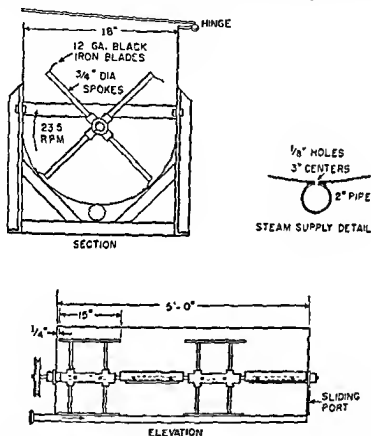
Fig. 11.—Cooker detail [*Courtesy of L. A. Underkofler, G. M. Severson K. J. Goering, and L. M. Christensen, Cereal Chem., 24 (No. 1) 1 (1947)*]

MIXING, STERILIZING, COOLING.—These operations were accomplished in a special cooker (refer to Fig. 11), which consisted of a covered mixer with facilities for injecting steam. The mixer was 18 in. in diameter and 5 ft. long. It was nondirectional and when operated at a speed of 23 r.p.m. agitated the bran mixture efficiently. A row of jets at the bottom of the cooker admitted steam for sterilization.

During operation, the bran (in 50-lb. batches) was mixed with an acid-salt solution in the special mixer, "sterilized" by the injection of steam for 30 min., and agitated continuously. A final moisture content of

[1] UNDERKOFLER, SEVERSON, GOERING, and CHRISTENSEN, *loc. cit.*

about 51 per cent was obtained by Underkofler and his collaborators[1] by adding 1 part of acid (0.2 N HCl) containing double the desired amount of mineral salts to 2 parts of bran. Previous studies had established the fact that practical sterility of the acid-moistened bran could be obtained by heating it at 93 to 99°C. for 15 to 30 min.; that butyric acid bacteria, a bad type of contaminant, could be eliminated; and that the few surviving mold spores caused no serious difficulties.

Cooling was accomplished by blowing air through the mass, with the mixer in operation, until its temperature reached about 35°C.

The moist bran was inoculated by blowing a spore culture onto the mass and continuing agitation of the mixer.

INOCULUM DEVELOPMENT.—The inoculum, or spore culture, was prepared in special galvanized iron pans, measuring 24 by 35 by 4 in. and equipped with covers containing a central air inlet and air outlets at each corner (refer to Fig. 12). These pans were especially designed to provide adequate aeration of the culture, optimum thickness of the bran layer, and some control of the humidity.

Each pan received 4.5 lb. of the standard corn-bran-acid mixture (refer to p. 70), which was uniformly distributed to form a layer about 0 5 in deep The pans and their contents were sterilized with steam and cooled The moist bran was then inoculated with mold spores and
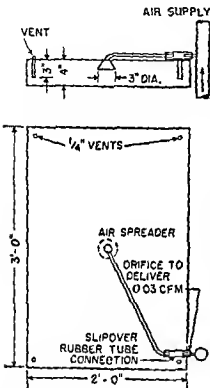
Fig. 12.—Inoculum pan. (*Courtesy of L. A. Underkofler, G M. Severson, K J Goering, and L. M. Christensen, Cereal Chem.* 24 (No. 1) · 1 (1917).]

incubated at 32°C. in a constant temperature cabinet. Air at a rate of 1,200 to 1,800 ml. per min. and at a temperature of 30 to 32°C. was admitted to each tray through the central inlet. When sporulation was at an optimum, usually at the end of 4 or 5 days, the cover of the pan was removed and the culture was allowed to air dry in the cabinet during a period of 24 hr.

INOCULATION METHODS.—Underkofler and his coworkers[1] studied the uses of mycelium and of spore inoculums. Mycelium inoculums could be used, but were inconvenient to handle and tended to become too contaminated for further use after two or more transfers. Spore inoculums

[1] *Ibid.*

proved to be very satisfactory and as little as 0 04 per cent of dry spore culture could be used successfully. However, an inoculum consisting of 0.1 per cent of dry spores was used for subsequent pilot-plant studies in order to provide a reasonable factor of safety against variations in spore viability.

INCUBATION METHODS.—Underkofler, Severson, Goering, and Christensen[1] studied extensively a number of different incubation methods in their search for a pilot-plant procedure that would be rapid and practical They found that the tray method was simple to employ, but that there
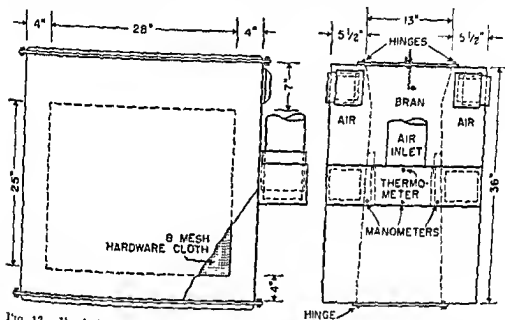
FIG 13—Vertical incubation cell. [*Courtesy of L. A. Underkofler, G. M Severson, K J Goering, and L. M. Christensen, Cereal Chem., 24 (No. 1) 1 (1947)*]

were problems associated with the use of the large number of trays required. In this method, it was not efficacious to pile the inoculated bran in layers over 2 in. deep in the pans, for the mold did not grow throughout the mass in the deeper layers Since the temperature of the bran generally commenced to rise in about 5 to 6 hr. after its inoculation, it was necessary at this point to commence aeration of the mass with air nearly saturated with moisture.

Studies were carried out using specially designed incubation cells and forced aeration. Vertical and inclined cells (refer to Figs 13 and 14), equipped with hardware cloth faces, proved to be satisfactory It was found that the temperature of incubation and the moisture content of the bran were dependent variables. The mold grew most rapidly in the bran

¹ *Ibid*

when the moisture content was adjusted suitably, between 32 and 38°C. The optimum moisture content resulted when 8 parts of 0.1 N hydrochloric acid were used with 10 parts of bran (by weight). Under these conditions a bran with an initial moisture content of 12 per cent contained

Fig. 14.—Inclined incubation cell. [Courtesy of L. A. Underkofler, G. M. Severson, K. J. Goering, and L. M. Christensen, Cereal Chem., 24 (No 1): 1 (1947).]

about 51 per cent of moisture at the time it was placed in the incubator. Two-stage incubation appeared to be advantageous.

DRYING.—Mold bran from the incubation cells, containing 25 to 30 per cent of moisture, may be dried in layers up to 4 ft. in depth in bins constructed with false bottoms through which dry air is blown. Underkofler and his associates[1] have pointed out that the air temperatures should not be in excess of about 45°C. (113°F.) if serious losses in amylolytic activity were to be avoided.

[1] Ibid

Semicommercial Production Methods.—An installation for producing mold bran on a semicommercial scale was constructed at the alcohol plant of the Farm Crops Processing Corporation (Omaha, Neb). A cooker of similar design but somewhat larger than the one described on p 73 was used. Primary incubation was carried out in inclined cells, secondary incubation in closed bins containing bottoms of perforated metal. Difficulties were encountered in connection with the usage of the primary incubation cells, for air control was found to be critical and temperature
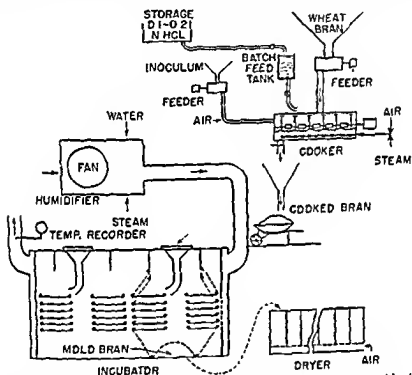
Fig 15 —Flow sheet for semicommercial mold-bran production [*Courtesy of L A Under-koffer, G M Severson, K J Goering, and L H Christensen, Cereal Chem, 24 (No 1) 1 (1947)*]

control very difficult to achieve. In addition, shrinkage of the bran created air leakages at the top of the cells. The mold bran produced in this unit with careful control was fair in quality.[1]

While the first unit was in production, a successful procedure for handling trays was devised, which led to the construction of a new incubator room (refer to Fig 15). Trays, measuring about 5 by 11 ft, were hinged lengthwise in such a manner that the front could be dropped down for discharging mold bran and raised for filling purposes. The trays were suspended in a special incubator room constructed of tile and supplied

[1] *Ibid.*

with air ducts for circulating humidified air at the proper temperature. The secondary incubator, previously used in the first unit, was employed to dry the mold bran. The difficulties concerned with control were absent in this unit. Likewise, mold growth was rapid and the quality was uniformly good.[1]

Commercial Production Method.[2]—Wheat bran and dilute hydrochloric acid (0.1 to 0.2 N) are mixed and heated to 100°C. with direct steam, at which temperature the mixture is held for 30 min. with continuous agitation. The temperature of the cooked bran is reduced to 32.2°C. (90°F.) through the use of a stream of air. Spores of *A. oryzae* are thoroughly mixed with the bran, which contains approximately 50 per cent moisture and has a pH of about 3.5. The inoculated bran is distributed rather uniformly on swinging trays (arranged to facilitate easy filling and removal of the mold bran) in special large-sized incubators. Humidified air at the proper temperature is caused to flow over and under the trays until the maximum amount of mold growth has taken place, which generally requires about 36 hr. The mold bran thus prepared is dropped from the trays and air-dried to a moisture content of approximately 12 per cent. One commercial product is sold under the trade name of "Eaglezyme."

Preparation and Saccharification of Mashes.—Mashes may be prepared with or without the use of acids, enzymes, and steam under pressure. A description follows of some of the methods now used in the laboratory or on a commercial basis.

Method of Hao, Fulmer, and Underkofler.[3]—Sixty grams of corn meal and 300 ml. of 0.04 N hydrochloric acid are placed in a 500-ml. Erlenmeyer flask and thoroughly mixed. The starch of the corn is gelatinized by heating the mixture (with occasional agitation) over a low flame or on a hot plate.

The mash is then placed in an autoclave and treated with steam at a pressure of 20 lb. per sq. in. for 30 min. The pH of the cooked and sterilized mash is adjusted to 4.5 to 5.0 with sodium hydroxide, sodium carbonate, or calcium carbonate. The mash, at a temperature of 30°C., is placed in a mixer and mold bran is added as a slurry in water. After agitation for 1 min., the mixture is returned to the 500-ml. Erlenmeyer flask and incubated for 1 hr. at 30°C. for saccharification of the soluble starches. A higher temperature (55°C.) and varying times (1 to 3 hr.) were also used in some experiments for conversion. (Hao and his associates inoculated a mash of the size mentioned above with 20 ml. of a

[1] *Ibid.*

[2] BOYER and UNDERKOFLER, *loc. cit.*

[3] HAO, FULMER, and UNDERKOFLER, *loc. cit.*

24-hr. culture of a special strain of *S. cerevisiae* in 10 per cent malt extract broth.)

Method of Roberts, Laufer, Stewart, and Saletan.[1]—Two different types of mashes were used by Roberts and his associates. These are described below under the headings of pressure mashing and atmospheric mashing.

PRESSURE MASHING.—To 400 ml. of water which has been heated to 50°C. are added 97 g. of wheat and 0.5 g. of dry mold bran The mash is held, with constant stirring, at 50°C. for 15 min. and then the temperature is raised during a 15-min. period to 66°C. at which temperature it is held for 30 min. The temperature of the mash is then raised to 93°C. during a 15-min period and held at 93°C. for 30 min. The mash is heated finally with steam for 1 hr. at a pressure of 20 lb. per sq. in and cooled to the temperature of conversion.

ATMOSPHERIC MASHING.—This procedure is identical with that of pressure mashing up to the point where the temperature reaches 66°C The mash is held at 66°C. for 55 min and cooled rapidly (within 5 min) to the saccharification temperature.

Saccharification is carried out at either 30 or 52.5°C. Mold bran (Polidase-C) is prepared as a slurry in a concentration of 2.5 to 4 per cent —the combined weight of grain and saccharifying agent is always maintained at 100 g.—and held for 15 min. at the saccharification temperature. The slurry is then added to the mash and saccharification effected by holding it for 30 min. at the specified saccharification temperature.

Commercial-scale Method.—Underkofler, Severson, and Goering[2] have reported on the use of mold bran on a commercial basis at the alcohol plant of the Farm Crops Processing Corporation, Omaha, Nebraska. The method used for preparing and saccharifying the mashes was as follows:

The grain is ground and prepared as a slurry, using 15 gal. of liquid per bushel of grain, at 43°C. (110°F.). The slurry is passed through precookers where the temperature is raised to approximately 66°C. (150°F.) by the direct injection of steam. It is then cooked for 5 min. at 166°C. (330°F.) at a gauge pressure of 120 lb. The temperature of the cooked mash is reduced to 77°C. (170°F.) by flashing the mash through two pressure-reducing chambers, one at a gauge pressure of 16 lb. and the other under vacuum.

The mold bran is prepared with cold water as a slurry containing the correct concentration (usually 4 per cent) for saccharification. Next the slurry is mixed into a stream of hot mash, reducing the temperature of the mixture to 61°C. (142°F.). The mixture is held for approximately 2 min.

[1] ROBERTS, LAUFER, STEWART, and SALETAN, *loc. cit.*
[2] UNDERKOFLER, SEVERSON, and GOERING, *loc. cit.*

at this temperature and is then pumped through coolers to the fermenters where it enters at a temperature of about 28°C. (82°F.). It requires about 3 hr. to fill a fermenter of 130,000-gal. capacity. About 30 min. after filling has begun, yeast is pumped into the fermenters. The temperature is maintained at a maximum of 35°C. (95°F.) in the fermenter by the continuous circulation of the mash through external coolers. Thus saccharification takes place largely in the fermenter where the initial pH is about 5.2 and the concentration 22.4 g. per 100 ml., or 30 gal. of liquid per bushel of grain.

Effect of Conditions of Saccharification on Alcohol Yields.—The method used in preparing the grain mashes, the pH and concentration of the mashes during saccharification, the nature and concentration of the mold bran used, the temperature and time of saccharification, and other factors, exert an important influence on the subsequent fermentation and yields of alcohol obtained. Several of these factors have already been discussed in the preceding paragraphs. Some additional information will be presented now.

Roberts and his coworkers[1] found that maximum alcohol yields were obtained with mold-bran saccharification when the mashes were cooked at atmospheric pressure.

Naturally there is no one pH value that is optimum under all conditions of saccharification. However, the use of a pH somewhere in the range of 4.5 to 5.0 is usually suitable for saccharification, as it favors particularly the activities of the amylolytic enzymes, α-amylase and β-amylase.

The concentration of mash at the time of saccharification depends upon the method used. The weight of grain usually represents about 16 to 20 per cent of the weight of the total mash.

Hao and his associates[2] concluded that saccharification of corn mashes for 1 hr. at 30°C. with mold-bran preparations was as satisfactory as employing 1 to 3 hr. at 55°C. (Refer to Table 11 on page 70.)

The usual concentration of mold bran employed for saccharification ranges from 2.5 to 4.0 per cent, depending upon the amylase activity of the product. A 4.0 per cent concentration based on the weight of grain used appears to produce most satisfactory results in terms of yields of ethyl alcohol.

Roberts and his collaborators[1] showed that mold-bran saccharification at 30°C. was equal to or slightly better than at 52.5°C. both in the case of mashes cooked at atmospheric pressure (66°C.) and in the case of those cooked with pressure (125°C.). They also demonstrated that the pre-

[1] ROBERTS, LAUFER, STEWART, and SALETAN, loc. cit.

[2] HAO, FULMER, and UNDERKOFLER, loc. cit.

malting of mashes with barley malt increased their liquefaction but did not result in improving the yields of alcohol over those saccharified with only 3 to 4 per cent mold bran.

Comparison of Mold Bran with Malt as a Saccharifying Agent.—The most suitable means for evaluating amylolytic agents is based upon comparisons of the yields of ethyl alcohol recovered from the saccharified grains. A number of such comparisons have been made. A few typical results and conclusions are included below.

Table 12 shows the results of plant-scale tests of mold bran at the Farm Crops Processing Corporation, Omaha, Neb. In the tests cited, mold bran was used as a replacement of supplemental malt in preparing yeast culture mashes, with the result that the yields of alcohol were increased. This use of mold bran has resulted also in a reduction of the time required for lactic souring (i.e., acid production by lactic acid bacteria) and higher yeast counts.[1]

TABLE 12.—RESULTS OF PLANT-SCALE TESTS OF MOLD BRAN[1]

No. of fermenters	Per cent of saccharifying agent in fermenter mashes	Per cent of saccharifying agent in yeast culture mashes	Av. alcohol yield/standard bu.	
			Proof, gal	190-proof, gal
299	10 malt	22 malt	4 77	2 51
817	9-10 malt	8 6 malt + 1 3 mold bran	5 17	2 72
6	4 mold bran	8 6 malt + 1 3 mold bran	5 21	2 76
12	9-10 malt	8 6 malt + 4 3 mold bran	5 15	2 71
7	3 9 6 2 malt + 2 2 9 mold bran	8 6 malt + 1 3 mold bran	5 26	2 77
12	9 10 malt	8 6 malt + 1 3 mold bran	5 23	2 75

[1] UNDERKOFLER, L. A., G. M. SEVERSON and K. J. GOERING, *Ind. Eng. Chem.* **38**, 980 (1946).

Table 13 shows results obtained by Roberts and his associates using different quantities of malt and mold bran as the saccharifying agents for corn, two kinds of wheat, and two kinds of granular wheat flour. The mashes were prepared as indicated in the previous section which was concerned with the preparation and saccharification of mashes.

A Comparison of Mold Preparations as Conversion Agents.—Hao and Jump[2] have compared some crude, intermediate, and purified mold preparations for their conversion efficiencies and yields of ethanol from corn mash. The preparations used by them are listed in Table 14. The

[1] UNDERKOFLER, SEVERSON, and GOERING, *loc. cit.*

[2] HAO, L. C., and J. A. JUMP, *Ind. Eng. Chem.* **37**: 521 (1945).

TABLE 13.—YIELD OF ALCOHOL IN *PROOF GALLONS* PER 100 LB. OF GRAIN ON DRY BASIS[1,2]

Saccharifying agent	Conversion temp. °C	Corn Pressure	Winter wheat Pressure	Winter wheat Atmosphere	Spring wheat Pressure	Spring wheat Atmosphere	Winter granular wheat flour Pressure	Winter granular wheat flour Atmosphere	Spring granular wheat flour Pressure	Spring granular wheat flour Atmosphere
8% malt	57 5	10 37	10 05	9 37	9 68	9 53	11 69	11 19	11.58	10 90
1% malt[3] + 2% mold bran	52 5	10 36	8 92	10 45	9 64	10 09	11 89	12 25	11 67	12 17
	30 0	10 34	9 57	10 35	9 80	10 10	11 35	12 39	11 87	12 42
2.5% mold bran	52 5	10 12	7.75	9 52	8 85	10 22	11 02	12 15	11 21	12 13
	30 0	10 43	9 58	9 82	9 38	10 18	11.19	12 22	11 56	12 43
3% mold bran	52 5	10 50	7 96	9 10	9 24	10.55	.....	12 42		12 39
	30 0	..		10 34						12 24
4% mold bran	32 5			10 17		9 97		12 40		12 70
	30 0	10 24		9 83		9 20		11 06		11 68
3% wet mold bran[4]	32 5	0 93		10 25		9 73		12 47		12 35
	30 0	10 24		9 83		9 20		11 06		11 68

[1] ROBERTS, M., S LAUFER, E D STEWART, and L T SALETAN. *Ind. Eng. Chem.*, 36: 811 (1944).

[2] Yields are corrected for alcohol produced from malt. Alcohol from mold bran is negligible. All yields are averages of two or more results

[3] For premalting

[4] Sample contained 50 per cent moisture, therefore, 6 g were used.

TABLE 14.—SOURCES OF MICROBIAL AMYLASE PREPARATIONS[1]

Name of Sample	Producer
Bacterial amylase I	[2]
Bacterial amylase II	Wallerstein Co., Inc.
Bacterial amylase III	[2]
Mold bran I	Frederick Stearns & Co.
Mold bran II	Wallerstein Co., Inc.
RHozyme	
Maltase No. 20	
RHozyme Intermediate	Rohm & Haas Co., Inc.
Maltase Intermediate	
Converzyme No. 503	
Converzyme No. 505	Joseph E. Seagram & Sons, Inc. (lab preparation)
Converzyme No. 511	[2]
Polidase-C	
Polidase-S	Schwarz Lab., Inc.
Diatane type 1211	
Diatane B-10	Jeffrey Lab.
Taka-Diastase	Parke, Davis & Co.
Alase	Takamine Lab.
Crystazyme	Frederick Stearns & Co.

[1] HAO, L C. and J A. JUMP. *Ind Eng Chem.*, 37, 521 (1945).

[2] Name of producer not revealed because of limited supply of product or other technicalities

mashes contained, in addition to the conversion agents, 91 parts of corn and 1 part of liquefying material (malt), unless otherwise indicated.

As a result of their studies, Hao and Jump made the following conclusions: All the crude mold-amylase preparations studied (Converzymes 503, 505, and 511; mold brans I and II; Diatane T-1211; and Polidase-C) were efficient agents for converting corn mash, the conversion efficiency being higher than that of malt but the yields of alcohol being about the same. The intermediate mold-amylase preparations were excellent agents for conversion and produced thinner mashes than malt and crude amylase preparations. The conversion efficiencies and yields of alcohol (based on total grain) were higher with 4 parts of RHozyme Intermediate or 6 parts of Maltase Intermediate with 8 parts of malt. The purified mold-amylase preparations (RHozyme, Maltase No 20, Taka-Diastase, Polidase-S, Alase, and Crystazyme) were also excellent conversion agents, 0.25 to 1.5 parts of the agents producing satisfactory conversion.

The value of mixing malt with mold-amylase preparations may be ascertained by reference to Table 15. The value of hydrochloric acid as a

TABLE 15—COMPARISON OF ALCOHOL YIELDS FROM CORN-MASH-CONVERTED PREPARATIONS AND 1 PER CENT MALT[1]

Run No	Conversion material	Mash composition, wt ratio[2]		Conversion efficiency, per cent	Yield, proof gal bu., based on	
		Conversion material	Liquefying material (malt)		Corn	Total grain
631	Malt	8	1	83 6	5 86	5 73
702	Diatane T-1211 (control)	5	1	90 0	5 93	5 74
731	Diatane T-1211 Malt	5 1	1	91 0	6 20	5 94
711	Diatane B-10 Malt	5 1	1	91 6	6 21	5 98
733	Diatane B-10	5	3*	96 5	6 37	6 15

[1] Hao, L. C. and J A Jump Ind Eng Chem., 37, 521 (1945)
[2] 91 parts corn in each case
* Concentrated hydrochloric acid

liquefying agent is also indicated   (The disadvantage of using acid for this purpose lies in the fact that the mash must be neutralized before the conversion agent is added.)

Hao and Jump reported that the addition of the mold-amylase

[1] Hao, L. C., and J A Jump, Ind Eng Chem., 37: 521 (1945)

TABLE 13.—YIELD OF ALCOHOL IN PROOF GALLONS PER 100 LB. OF GRAIN ON DRY BASIS[1][2]

Saccharifying agent	Conversion temp. °C	Corn Pressure	Winter wheat Pressure	Winter wheat Atmospheric	Spring wheat Pressure	Spring wheat Atmospheric	Winter granular wheat flour Pressure	Winter granular wheat flour Atmospheric	Spring granular wheat flour Pressure	Spring granular wheat flour Atmospheric
8% malt	57.5	10.37	10.05	9.37	9.68	9.53	11.69	11.19	11.58	10.90
1% malt[3] + 2% mold bran	52.5	10.36	8.92	10.45	9.04	10.09	11.89	12.25	11.67	12.17
	30.0	10.34	9.57	10.35	9.80	10.10	11.35	12.39	11.87	12.42
2.5% mold bran	52.5	10.12	7.76	9.52	8.85	10.22	11.02	12.15	11.21	12.13
	30.0	10.43	9.58	9.82	9.38	10.18	11.19	12.22	11.66	12.43
3% mold bran	52.5	10.50	7.96	10.10	9.24	10.35	....	12.42	..	12.39
	30.0			10.34			...			12.24
4% mold bran	52.5			10.17		9.97		12.40		12.70
	30.0			10.25		9.73		12.47		12.35
3% wet mold bran[4]	52.5	9.93		10.25		9.73		12.47		12.35
	30.0	10.24		9.83		9.20		11.98		11.68

[1] Roberts, R. M., S. Lauter, E. D. Stewart, and L. T. Saletan, Ind Eng Chem, 36·811 (1944).
[2] Yields are corrected for alcohol produced from malt. Alcohol from mold bran is negligible. All yields are averages of two or more results.
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Maltase No 20	
R Hozyme Intermediate	Rohm & Haas Co., Inc.
Maltase Intermediate	
Converzyme No 503	
Converzyme No 505	Joseph E Seagram & Sons, Inc. (lab preparation)[2]
Converzyme No 511	
Polidase-C	
Polidase-S	Schwarz Lab., Inc.
Diatane type 1211	
Diatane B-10	Jeffrey Lab.
Taka-Diastase	Parke, Davis & Co.
Alase	Takamine Lab.
Crystazyme	Frederick Stearns & Co.

[1] Hao, L. C., and J. A. Jump, Ind. Eng. Chem., 37, 521 (1945).
[2] Name of producer not revealed because of limited supply of product or other technicalities.

mashes contained, in addition to the conversion agents, 91 parts of corn and 1 part of liquefying material (malt), unless otherwise indicated.

As a result of their studies, Hao and Jump made the following conclusions: All the crude mold-amylase preparations studied (Converzymes 503, 505, and 511; mold brans I and II; Diatane T-1211; and Polidase-C) were efficient agents for converting corn mash, the conversion efficiency being higher than that of malt but the yields of alcohol being about the same. The intermediate mold-amylase preparations were excellent agents for conversion and produced thinner mashes than malt and crude amylase preparations. The conversion efficiencies and yields of alcohol (based on total grain) were higher with 4 parts of Rllozyme Intermediate or 6 parts of Maltase Intermediate than with 8 parts of malt. The purified mold-amylase preparations (Rllozyme, Maltase No. 20, Taka-Diastase, Polidase-S, Alase, and Crystazyme) were also excellent conversion agents, 0.25 to 1.5 parts of the agents producing satisfactory conversion.

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		Conversion material	Liquefying material (malt)		Corn	Total grain
631	Malt	8	t	88 6	5 86	5 73
702	Diatane T-1211 (control)	5	t	90 0	5 93	5 74
731	{Diatane T-1211 Malt	5} 1}	1	91 0	6 20	5 91
741	{Diatane B-10 Malt	5} t}	t	91 6	6 21	5 98
733	Diatane B-10	5	3[a]	96 5	6 37	6 15

[1] Hao L C and J A Jump *Ind Eng Chem*, 37, 521 (1945)
[2] 91 parts corn in each case
[a] Concentrated hydrochloric acid

liquefying agent is also indicated. (The disadvantage of using acid for this purpose lies in the fact that the mash must be neutralized before the conversion agent is added)

Hao and Jump[1] reported that the addition of the mold-amylase

[1] Hao, L. C., and J A Jump, *Ind Eng Chem*, 37: 521 (1945).

preparation at intervals increased the efficiency of conversion and the yield of ethyl alcohol by 1 to 2 per cent. About three-fifths of the preparation was added at the conversion time and the remaining portion after the fermentation had proceeded for 20 hr.

SUBMERGED-CULTURE PRODUCTION OF MOLD AMYLASES

Considerable interest has been shown recently in the production of fungal amylases by submerged-culture methods. These enzyme products are manufactured on the premises for use as a malt substitute or supplement in the conversion of grains for such purposes as the production of industrial alcohol. The amylases are generally used without concentration or purification and may be produced on a number of media.

Production.—The production of fungal amylases by submerged-culture methods has been studied by Balankura, Stewart, Scalf, and Smith;[1] Van Lanen and Le Mense;[2] Le Mense, Corman, Van Lanen, and Langlykke;[3] Adams, Balankura, Andreasen, and Stark;[4] and others.

Production involves growing a high amylase-producing strain of mold in a suitable medium under controlled conditions of temperature, pH, and aeration.

Organisms Used.—Van Lanen and Le Mense,[2] and Le Mense and associates[3] studied the production of amylases in a thin stillage medium by 367 cultures of molds, which included species of the genera Penicillium, Aspergillus, Rhizopus, Mucor, and Monilia. Only 10 per cent of the 80 cultures of the genus Penicillium examined demonstrated dextrinizing activity and this was of low order. Cultures of the genera Rhizopus, Mucor, and Monilia produced either very little or no dextrinizing enzyme. Greatest activity was observed in cultures of the genus Aspergillus. Of 278 cultures studied, 34 produced the dextrinizing enzyme. Highest concentrations of this enzyme were produced by strains of A. niger, A. oryzae, A. alliaceus, A. foetidus, and A. wentii. Some strains of A. niger produced both dextrinizing and saccharifying enzymes. Among these was A. niger NRRL 367 which exhibited the greatest potency of any of the cultures examined by the Northern Regional Research Laboratory group.

Media.—Various media may be used for the production of fungal amylases by submerged-culture methods. Le Mense and his associates[5]

[1] Balankura, B., F. D. Stewart, R. E. Scalf, and L. A. Smith, Jour. Bact., 51 (No. 5), 594 (1946).

[2] Van Lanen, J. M., and E. H. Le Mense, Jour. Bact., 51 (No. 5), 595 (1946).

[3] Le Mense, E. H., J. Corman, J. M. Van Lanen, and A. F. Langlykke, Jour. Bact., 54 (No. 2), 149 (1947).

[4] Adams, S. L., B. Balankura, A. A. Andreasen, and W. H. Stark, Ind. Eng. Chem., 39, 1615 (1947).

[5] Le Mense, Corman, Van Lanen, and Langlykke, loc. cit.

· used a medium that contained thin stillage (a product of the alcoholic fermentation of corn and sorghums), 2 per cent of corn meal, and 0.5 per cent of calcium carbonate. The culture liquors obtained after growth of the mold for 3 to 5 days with continuous aeration were satisfactory substitutes for malt in the conversion of corn mashes.

The relative values of other media for the production of the dextrinizing enzyme by *A. niger* NRRL 337 are indicated in Table 16.

TABLE 16.—THE PRODUCTION OF DEXTRINIZING ENZYME BY *Aspergillus niger* NRRL 337 CULTIVATED IN VARIOUS MEDIA[1,2]

Protein source	Carbohydrate source	Concentration of dextrin-izing enzyme, units/ml
Corn steep liquor, 3%	None	2 2
Corn steep liquor, 3%	Glucose, 2%	8 2
Corn steep liquor, 3%	Molasses, 2%	4 6
Corn steep liquor, 3%	Corn meal, 2%	10 2
Dried tankage, 2%	None	2 1
Dried tankage, 2%	Glucose, 2%	9 3
Dried tankage, 2%	Molasses, 2%	11 5
Dried tankage, 2%..	Corn meal, 2%	8 7
Soybean meal, 2%	None	7 0
Soybean meal, 2%	Glucose, 2%	7 4
Soybean meal, 2%.	Molasses, 2%	8 5
Soybean meal, 2%.	Corn meal, 2%	11 2
Thin stillage	None	1 7
Thin stillage	Glucose, 2%	11 5
Thin stillage	Molasses, 2%	7 9
Thin stillage	Corn meal, 2%	16 5
Thin stillage	Xylose, 2%	5 3
Thin stillage	Lactose, 2%	6 7
Thin stillage	Sucrose, 2%	11 0
Thin stillage	Maltose, 2%.	14 5

[1] LE MENSE E. H. J. CORMAN J. M. VAN LANEN and A. F. LANGLYKKE *Jour. Bact.* 54 (No. 2) 149 (1947).

[2] Enzyme determinations were made after cultures were shaken for 5 days.   Composition of medium protein and carbohydrate as shown plus 0.5 per cent of calcium carbonate.

Adams, Balankura, Andreasen, and Stark[1] employed a medium that contained 5 g per 100 ml of distillers' dried solubles (stillage from the distillation column, screened to separate out most of the suspended solids, evaporated to about 30 per cent solids and then dried on drum driers) and 1 g of corn meal per 100 ml   Distillers' dried solubles proved to be

[1] ADAMS BALANKURA ANDREASEN and STARK *loc. cit.*

better than stillage for mold amylase production. The pH of this medium was adjusted to 5.0 with calcium carbonate. Adams and his collaborators reported that the mold amylase produced in this medium could be used instead of barley malt, and that the yield of ethyl alcohol was actually 0.2 to 0.3 proof gal. per bu. of grain greater than when malt was employed.

Conditions of Production.—Le Mense and his associates[1] dispensed 50-ml. amounts of medium in 200-ml. flasks and 200-ml. amounts in 1-liter flasks. These were sterilized with steam for 30 min. at 20 lb. pressure. After cooling and inoculation, they were incubated at 30°C. and shaken continuously in a Kahn-type shaker (ninety 3-in. strokes per min.). 4-liter amounts of medium were used for aeration studies in 8-liter Pyrex cylinders, which were equipped with air spargers built of perforated aluminum tubing and had aluminum plate lids. These containers plus their contents were sterilized for 1 hr. at 25-lb. pressure.

Adams and his collaborators[2] sterilized 1-liter portions of their medium in 2-liter, cone-shaped flasks. In building up the inoculum, the medium was inoculated the first time with 2.0 per cent by volume of a sporulated culture (grown by surface method) of *A. niger* NRRL 337. The flask was incubated at 30°C. in a water bath and the contents were aerated for 24 hr. with 0.2 cu. ft. per min. per liter of air, using an Aloxite sparger. Thereafter the mold culture was transferred serially through a number of 24-hr. submerged stages (usually two), using 0.5 per cent by volume of inoculum for each succeeding mash. The final stage was aerated for 48 hr.

Methods Used for Evaluating Submerged Fungal Amylase Preparations.—These are concerned with a determination of the dextrinizing and saccharifying activities of the enzymes and of the alcohol produced from grain mashes converted by them and subsequently fermented by yeasts. Adams, Balankura, Andreasen, and Stark[2] made three determinations: α-amylase activity by the 30°C. method of Sandstedt, Kneen, and Blish;[3] the saccharifying power by an unpublished method of Hao; and the alcohol yields by the method of Stark and his associates.[4] Le Mense and his associates[5] determined the dextrinizing activity by the method of Sandstedt, Kneen, and Blish[3] (as modified by Olson, Evans and Dickson[6]); and the maltase activity by calculating the increase in reduc-

[1] Le Mense, Corman, Van Lanen, and Langlykke, *loc. cit*

[2] Adams, Balankura, Andreasen, and Stark, *loc. cit.*

[3] Sandstedt, R. M , E. Kneen, and M J. Blish, *Cereal Chem*, 16: 712 (1939)

[4] Stark, W. H., S. L. Adams, R. E. Scalf, and P. Kolachov, *Ind. Eng. Chem.*, *Anal. Ed.*, 15: 443 (1943).

[5] Le Mense, Corman, Van Lanen, and Langlykke, *loc. cit*

[6] Olson, W. J., R. Evans, and A. D. Dickson, *Cereal Chem.*, 21: 533 (1944)

ing power, found by Somogyi's method[1] after incubating a 10-ml. portion of culture filtrate with 20 ml. of a 1.05 per cent maltose solution for 2 hr. at 30°C. (The mixture was kept at a pH of 4.6 by the addition of an "acetate buffer to the maltose solution ")

Mashing and Conversion Methods.—The method of Le Mense *et al*[2] was as follows: Into each of a series of 500-ml Erlenmeyer flasks were placed 49.5 g. of ground corn, 0.5 g. of ground barley malt, and 170 ml of tap water warmed to 70°C. The flasks were incubated in a water bath at 70°C. and the mashes were stirred intermittently for 30 min. for premalting. The mashes were then cooked for 30 min. at a steam pressure of 25 lb. per sq. in. and cooled to 75°C. The mold enzyme liquor was added to the mash with enough water to lower the temperature to 55 to 56°C., at which temperature conversion was carried out for 30 min. with frequent agitation of the mashes. The same method was used for malt controls, except that 45 g. of corn and 5 g. of malt were used, 0.5 g. of the latter being for premalting and 4.5 g. for conversion.

In the procedure of Adams and his associates,[3] the grain to be converted (corn) was cooked for 1 hr at atmospheric pressure, autoclaved for another hour at 22 lb. pressure, and cooled to the temperature of conversion. Sufficient submerged mold culture was added to the mash to represent 10 per cent of its final volume. The mash was agitated for 5 min. and cooled to the temperature of setting. Ten per cent by volume of stillage was then added, and the pH was adjusted to 4.8 and the total volume to 38 gal per bu. of grain. The mash was converted at a temperature of 145°F.

Fermentation.—The corn mashes converted by the method of Le Mense and coworkers were inoculated in the proportion of 2 per cent by volume with a 24-hr. culture of distillers' yeast (NRRL Y567), after being cooled to 30°C., the temperature of fermentation. The final volume in the fermentation flask was about 250 ml. in each case. The fermentations were allowed to continue for 72 hr. The fermented mashes (beers) were made up to a volume of 300 ml of which a 200-ml aliquot was distilled. In each instance 100 ml of distillate were collected. The alcohol content was ascertained by determination of the refractive index.

Yields.—Yields of alcohol obtained by the procedure of Adams and his associates varied from 6.16 to 6.27 proof gal per bu, as compared with 5.95 proof gal. per bu for the malt-converted control

[1] Somogyi, M., *Jour Biol Chem*, **160**: 61 (1945)
[2] Le Mense, Corman, Van Lanen and Langlykke, *loc cit*
[3] Adams, Balankura, Andreasen, and Stark, *loc cit*

Some Factors Affecting Amylase Production.—Erb, Wisthoff, and Jacobs[1] investigated some of the factors that influence amylase production by *A. niger* NRRL 337. The basic medium used by them was a 10 per cent corn mash, acidified to a pH of 4.0 with sulphuric acid and cooked for 1 hr. at a steam pressure of 30 lb. The concentration of the medium was adjusted as desired, and calcium carbonate was added to bring the pH to 5.3. Urea, in the proportion of 0.25 per cent, was added to each flask. The medium was distributed in 100-ml. amounts in 1-liter Erlenmeyer flasks, sterilized, cooled, and inoculated with the spores of *A. niger* NRRL 337 and shaken continuously at 30 to 32°C. for the desired period of time.

It was found that the addition of small quantities of sodium chloride or potassium chloride (0.001 g. per 100 ml.) stimulated amylase production.

Enzyme production was correlated with the concentration of the grain in the medium. When an incubation period of 96 hr. was used, the enzyme production of a medium with an initial concentration of 10° Brix was decided superior to that of media of 8 and 6° Brix concentrations, respectively. However, based on an incubation period of 22 hr., the medium of 6° Brix concentration possessed greater enzyme activity than the media of greater concentration.

Sodium pentachlorphenate (Dowicide G), in a concentration of 0.006 to 0.012 g. per 100 ml. of medium, and ammonium bifluoride, in a concentration of 0.015 to 0.02 g. per 100 ml. of medium, may be used to control bacterial contamination during amylase production by *A. niger* NRRL 337, according to Erb, Wisthoff, and Jacobs. Sodium pentachlorphenate tends to inhibit or prevent sporulation of the mold. However, low amylase production and sporulation in shake-flask cultures are frequently correlated.

Action of Mold Enzymes in the Saccharification of Starch.—Corman and Langlykke[2] studied the action of the amylolytic enzymes found in mold culture filtrates on the saccharification of starch. The action appeared to be due principally to an α-amylase and to a glucogenic enzyme system. Little or no β-amylase was observed in the mold culture filtrates. The action of the fungal α-amylase was related to that of malt α-amylase. The α-amylase liquefied starch and formed dextrins and maltose. The glucogenic enzyme system acted upon maltose, dextrins, and starch and formed glucose. This system differed markedly from β-amylase which produces maltose. The efficiency of saccharification

[1] Erb, N. M., R. T. Wisthoff, and W. L. Jacobs, *Jour. Bact.*, 55 (No. 6): 813 (1948).

[2] Corman, J., and A. F. Langlykke, *Cereal Chem.*, 25 (No. 3): 190 (1948).

appeared to be more closely correlated with the activity of the glucogenic enzyme system than with the activity of α-amylase.

USE OF BACTERIAL AMYLASE PREPARATIONS AS LIQUEFYING AGENTS

Bacterial amylase preparations may be used instead of malt for the thinning of cooked grain mashes in the production of ethyl alcohol, according to Hao and Jump [1]. Table 17 compares alcohol yields from

TABLE 17.—COMPARISON OF ALCOHOL YIELDS FROM CORN MASH LIQUEFIED WITH BACTERIAL AMYLASE PREPARATIONS[1]

Run no	Liquefying material	Weight ratio			Conversion efficiency, per cent	Yield,[2] proof gal/bu. based on	
		Liquefying material	Converzyme No 505	Corn[3]		Corn	Total grain
361	Malt (control)[4]	1 0	8	91	91 4	6 03	5 66
362	Bacterial amylase I	1 0	8	91	91 5	6 04	5 72
363	Same	1 5	8	91	92 0	6 14	5 77
312	Same	1 0	5	91	91 0	6 02	5 79
313	Same	0 5	5	91	86 8	5 72	5 53
432	Bacterial amylase II	1 0	8	91	93 7	6 18	6 00
433	Same	1 5	8	91	93 5	6 17	5 99
442	Bacterial amylase III	1 0	8	91	92 9	6 11	5 78
413	Same	0 5	8	91	91 5	6 01	5 70
360	Conc HCl	3 0[5]	8	91	95 5	6 30	6 10
393	Same	3 0	8 (malt)	91	81 0	5 51	5 41

[1] Hao, L. C. and J. A. Jump, *Ind Eng Chem* **37** 521 (1945)
[2] Weight of corn in the mash is the same in all the fermentation runs this corn has a starch content of 61 8 per cent and a moisture content of 10 6 per cent
[3] All yields are calculated on a dry basis.
[4] The malt has a starch content of 50 0 per cent and a moisture content of 8 per cent
[5] The acidity of the mash during cooking is approximately 0 06 N The pH is adjusted to 5 6 before the addition of conversion material

corn mash in which the starch liquefying agents were malt, bacterial amylases, and concentrated hydrochloric acid, respectively, and in which the saccharifying agent was Converzyme No 505 It will be noticed that the alcohol yields from corn mash liquefied with bacterial amylases were slightly higher than that from the malt control when equal weights of the liquefying agents were used Actually, the bacterial amylases produced a mash of lower viscosity than the malt (based on the uses of equal weights of each), according to Hao and Jump Bacterial amylase III was a purified concentrate

[1] Hao and Jump, *loc. cit*

Table 17 also indicates that concentrated hydrochloric acid was the most efficient agent for liquefying the mash, under the conditions employed, the conversion efficiency being 95.5 per cent when the saccharifying agent was Converzyme No. 505.

THE AMYLO PROCESS

This process is primarily one for converting starch to sugar by the use of selected molds, some of which have the ability to produce small quantities of alcohol from sugar.

The grain to be hydrolyzed is soaked in water for several hours to soften it. It is then mixed with approximately twice its weight of water and heated at a pressure of 4 atmospheres to render the starch soluble. Acidification of the mash with 0.6 to 0.8 parts by weight[1] of hydrochloric or sulphuric acid per 100 parts of grain facilitates the liquefaction of the starch. The sterilized mash is cooled to 40 to 38°C. and inoculated with a pure culture of a *Mucor* or a related mold. *M rouxii, Rhizopus japonicus, R. tonkinensis,* and *R delemar* are some of the molds that have been used in the Amylo process. Sterile air is passed through the seeded mash for 24 hr., while the temperature is maintained at 38°C. The mash is then cooled to at least 33°C. and inoculated with yeast.

By using a modification of the Amylo process, developed by Boulard,[2] starch hydrolysis and fermentation are carried out simultaneously by adding at the same time *Mucor boulard* No 5 and a yeast. It is claimed that time is saved by the use of the Boulard process.

A MODIFIED AMYLO PROCESS

The use of a modified Amylo process for the saccharification of granular wheat flour mashes in the production of ethyl alcohol has been described by Erb and Hildebrandt.[3] In brief, this process involves the development of a mold inoculum, the preparation of the main mashes, the addition of the mold inoculum and a yeast starter (*Saccharomyces anamensis*) to the mash, and saccharification and fermentation.

Development of the Mold Inoculum.—Saccharification of the starch in the granular wheat flour mashes is brought about by the enzymes produced by the mold. In order to facilitate rapid saccharification of the mashes, it is necessary to build up a volume of inoculum equivalent to 6 to 12 per cent of the volume of the final mash. This is accomplished by

[1] Owen, W L , The Amylo Process of Malting in Whiskey Distilleries, *Am. Wine & Liquor Jour*, June, 1936

[2] Boulard, H , Société d'exploitation des procédés, H. Boulard, 1931

[3] Erb, N M., and F M Hildebrandt, *Ind Eng Chem.*, **38**: 792 (1946)

carrying the development through three stages, the first of which is initiated in the laboratory and the next two in the plant.

Laboratory Stage.—The mold used is a pure culture of either *Rhizopus delemar* or *R. boulard* and is carried on slants of malt agar. The first step in the development of the inoculum is the inoculation of a special medium in a 100-ml. Erlenmeyer flask with mold spores. The medium contains ingredients in the following proportions. 25 g. of flour, 0.1 g of ammonium sulphate, and 500 ml. of water. It is dispensed in shallow layers in 100-ml. Erlenmeyer flasks and sterilized at 250°F (121.1°C.) for 1 hr The spores produced in one flask are suspended in 3,000 ml. of water and used to inoculate the sterilized medium of the vessel employed in the first plant stage described below

Plant Stages.—A medium containing the following materials is sterilized for 1 hr. at 250°F (121 1°C.) in a jacketed steel pressure vessel which has a working capacity of 75 gal . 20 lb. of ground malt, 200 g. of ammonium sulphate, 5 g of zinc sulphate, 100 ml. of phosphoric acid, and sufficient water to make 75 gal. The mash is cooled to 90°F. (32.2°C.) and inoculated with the 3,000-ml. suspension of mold spores prepared in the laboratory The pH of the mash before inoculation is 3 8 to 4.0. The seeded mash is aerated at a pressure of 5 lb. Within a period of 20 hr., there is abundant growth of the mold and the culture is ready for use to inoculate the next larger medium

In the second plant stage, the medium is prepared and sterilized in a closed steel pressure vessel which has a working capacity of 2,500 gal. and which contains cooling coils The medium used in this vessel is 500 gal. of granular flour and malt from the mashing system of the plant, 300 gal of stillage, 0 5 lb. of aluminum powder, 20 g. of zinc sulphate, 6 lb. of ammonium sulphate, 1,000 ml. of sulphuric acid, and sufficient water to make approximately 2,500 gal The resultant pH of the medium is 3 8 to 4 2. It is sterilized at 250°F. for 1 hr and subsequently cooled to 95°F. (35°C.). The contents of the vessel from the first stage are blown into this vessel to serve as its inoculum. The seeded medium is aerated at about 5 lb pressure for 24 hr.

The aluminum powder is used as a constituent of the foregoing medium to counteract a somewhat toxic ingredient of the stillage which appears to inhibit growth of the mold The action of the interfering substance may be reduced by dilution and it may be removed by treatment with carbon

Preparation of Main Mashes. The mashes for conversion by malt and by molds are prepared in a similar manner, except for the conversion agent The one that is malt-converted contains 190 lb of flour and 20 lb of malt per 100 gal Two antiseptics are added to the mash—0 75 lb

of ammonium bifluoride and 0.7 lb. Dowicide G for each 1,000 gal. of mash—for no attempt is made to sterilize it by steam. The mash is converted in a mash tub at 145°F. (62.7°C.) for 1 hr. with agitation. The mash that is to be converted by the molds is prepared in the same manner, except that the amount of malt is 2 per cent.

Addition of Inoculums.—The control mash (malt saccharified) is cooled, pumped into the fermenter and 4 per cent of yeast inoculum (starter), based on total charge, is added. The amount of mash and yeast added initially fills about 30 per cent of the fermenter. This mixture is aerated for 3 to 4 hr. Then the tank is filled to 20,000 gal. by the addition of two more mashes prepared as described above.

The procedure with the mold inoculum is similar, except that the contents of the 2,500-gal. vessel containing the mold are added to the fermenter along with the yeast. Conversion and fermentation proceed simultaneously

Saccharification and Fermentation.—Saccharification and fermentation are complete in 40 hr. when the volume of mold inoculum represents 12 per cent of the final volume of the mash; and in 55 hr. when the volume of inoculum represents 6 per cent of the final volume of the mash.

Yields.—When mold was used as the principal conversion agent, the average yield from 10 fermenters was 12.1 proof gal. per 100 lb. of dry grain, which represented 91.2 per cent fermentation efficiency. When malt alone was employed, the average yield from 180 fermenters was 11.0 proof gal per 100 lb. of dry grain, which represented 84 per cent fermentation efficiency

ACID SACCHARIFICATION OF GRAINS AND OTHER STARCH-CONTAINING MATERIALS

Grains, vegetables, and other starch-containing materials may be saccharified through the use of acids, such as sulphuric or hydrochloric acids In general, the products to be converted are macerated, or ground, mixed with a measured quantity of acidulated water, and treated with steam under pressure.

After saccharification, the mashes may be adjusted to the desired pH level for fermentation with ammonium hydroxide, sodium hydroxide, sodium carbonate, or calcium carbonate. The use of ammonium hydroxide is particularly desirable, since the ammonia may act as a source of nitrogen for yeast growth If sulphuric acid is used for the conversion of the starch and subsequently neutralized with calcium carbonate, the precipitated calcium sulphate may be separated from the wort by sedimentation and/or filtration.

Severson[1] has investigated the saccharification of various grain mashes with hydrochloric acid. Maximum yields of ethanol from the acid-saccharified grains averaged somewhat lower than those obtained by malt saccharification. Highest yields of ethanol were obtained from corn when the mash was heated for 2 to 3 hr. at a steam pressure of 25 lb per sq. in. in the presence of 0.10 N hydrochloric acid [2]

TABLE 18—EFFECT OF MALT, SOYBEAN MEAL, AND MOLD BRAN UPON THE ALCOHOL YIELDS FROM ACID-SACCHARIFIED CORN MASH[1]

Malt and soybean meal			Mold bran and malt			Mold bran and soybean meal		
Proportion of material as per cent of corn		Yield, per cent	Proportion of material as per cent of corn		Yield, per cent	Proportion of material as per cent of corn		Yield, per cent
M	S		MB	M		MB	S	
0	0	76 8	0	0	75 3	0	0	84 0
1	0	76 4	2	0	82 5	1	0	87 1
2	0	76 0	3	0	82 9	2	0	88 6
3	0	75 5	5	0	82 9	3	0	89 8
						5	0	92 4
0	2	75 7	0	2	78 0			
0	3	78 5	2	t	83 0	0	3	87 8
0	5	77 0	3	1	82 2	1	1	90 9
t	1	78 5	5	t	82 5	2	1	90 5
1	2	78 3				3	1	90 7
t	3	76 4	2	2	81 2			
2	1	76 5	3	2	82 6	t	2	90 7
2	2	77 5	5	2	82 7	2	2	91 2
2	3	76 6				3	2	91 8

[1] SCHOENE L. E I FULMER and L. A UNDERKOFLER Ind Eng Chem 32 544 (1940)

Schoene, Fulmer, and Underkofler[2] have shown that normal yields of ethanol may be obtained by the addition of mold bran to acid-saccharified mashes. Their procedure for preparing acid-saccharified mashes, based on the optimum conditions found by Severson. was as follows. Measured quantities of corn meal and 0 1 N hydrochloric acid, 100 g and 500 ml , respectively, were placed in each 1,000-ml Erlenmeyer flask used and the mixture was cooked for 2 5 hr at a steam pressure of 25 lb per sq in   The pH of the cooked mashes was adjusted to 5 0 with concentrated

[1] SEVERSON, G , Iowa State Coll Jour Sci 11: 215 (1937)
[2] SCHOENE, L , E I FULMER, and L A UNDERKOFLER, Ind Eng Chem 32: 544 (1940)

ammonium hydroxide. Weighed portions of amylolytic (enzyme-containing) materials were added to the acid-saccharified mashes and final saccharification was carried out at 55°C. for 60 min. Results of typical experiments by Schoene, Fulmer, and Underkofler are shown in Table 18 on previous page.

An examination of Table 18 indicates that the best results were obtained with mold bran. Schoene and his associates suggested that the increased yields resulting from the use of mold bran were due to the large variety of enzymes that it contained. For example, mold bran contains emulsin, an enzyme that may hydrolyze gentiobiose (a disaccharide that may be formed during acid saccharification) to dextrose.

Mixtures of mold bran and soybean meal also produced increased yields of ethanol from acid-saccharified corn mashes.

According to Hayek and Shriner,[1] starch-containing mashes may be hydrolyzed (saccharified) satisfactorily by the use of sulphurous acid. They showed that pure starch may be completely converted to glucose in 15 min. at 165°C. in the presence of 0.2 to 0.4 per cent of sulphur dioxide; that corn mash may be hydrolyzed in 15 min. at 160°C. with 2 per cent of sulphur dioxide; and that wheat mash may be hydrolyzed in 10 min. at 165 to 170°C. with 2 per cent of sulphur dioxide. As high as 95 per cent of glucose is obtained from corn and wheat mashes by hydrolysis with sulphur dioxide. The sulphur dioxide may be removed from the hydrolyzed mashes and fermentation initiated. Yields of ethyl alcohol obtained from sulphurous acid-hydrolyzed mashes compare favorably with those secured from malt-converted mashes, according to Hayek and Shriner.[1]

The pH of a mash saccharified with acid must be adjusted before it is inoculated with yeast or other fermentation organism. Such adjustment of the pH usually increases the salt concentration of the mash.

ACID SACCHARIFICATION OF AGRICULTURAL RESIDUES

Dunning and Lathrop,[2] of the Northern Regional Research Laboratory, have reported on their research concerning the laboratory-scale saccharification of agricultural residues, such as corncobs, cottonseed hulls, flax shives, oat hulls, and sugar-cane bagasse.

The agricultural residues, typical analyses for which are given in Table 19, are hydrolyzed in two stages. During the first stage the pentosans are hydrolyzed by the use of dilute sulphuric acid, and during the second stage the cellulose is hydrolyzed by the use of concentrated sulphuric acid.

[1] Hayek, M., and R. L. Shriner, *Ind. Eng. Chem.*, 36 (No. 11): 1001 (1944).
[2] Dunning, J. W., and E. C. Lathrop, *Ind. Eng. Chem.*, 37 (No. 1): 24-29 (1945).

TABLE 19.—PERCENTAGE ANALYSIS OF REPRESENTATIVE HARDWOODS, SOFTWOODS, AND AGRICULTURAL RESIDUES[1]

Material	Pentosan	Cellulose	Lignin
Ponderosa pine[2]	7.4	52.3	26.6
Tanbark oak[2]	19.6	44.8	24.8
Corncobs. ..	28.1	36.5	10.4
Bagasse	20.4	41.3	14.9
Oat hulls	29.5	33.7	13.5
Cottonseed hulls	21.0	35.1	16.8
Flax shives.	23.0	38.0	21.0

[1] DUNNING, J. W. and E. C. LATHROP, Ind Eng Chem., 37 (No. 1) 24 (1945)
[2] RITTER, G. J., and L. C. FLECK, Ind Eng Chem., 14: 1050 (1922).

Pentosan Hydrolysis.—The purpose of this hydrolysis is to separate the pentosans as xylose and furfural without saccharifying the cellulose.

A study of the effects of acid concentration, temperature, time, and other factors upon the hydrolysis of pentosans has been made by Dunning and Lathrop[1] and it has been found that satisfactory hydrolysis may be accomplished under carefully controlled conditions. For example, when corncobs were treated with 1.9 per cent of sulphuric acid at a temperature of 121°C. for 50 min., using a solid to liquid ratio of 30:100, the yield of xylose was 86.1 per cent, that of furfural (as xylose) 9.3 per cent, and that of dextrose 0.78 per cent. Thus 95.4 per cent of the pentosans were hydrolyzed, whereas less than 1 per cent of dextrose was produced.   Data concerning other conditions found satisfactory for the separation of pentosans from hexosans may be found in the table which follows:

TABLE 20.—EFFECTS OF ACID CONCENTRATION, TEMPERATURE, AND TIME UPON SEPARATION OF PENTOSANS FROM HEXOSANS[1]

Acid concentration (sulphuric), per cent	1.9	4.1	4.4
Temperature, °C	121	100	100
Time, min	50	55	50
Solid : liquid ratio, g /100 ml	30:100	6:100	6:100
1 Yield of xylose, per cent	86.1	81.1	86.9
2. Yield of furfural (as xylose), per cent	9.3	10.9	8.4
3 Sum of 1 and 2	95.4	95.0	95.3
4 Yield of dextrose, per cent	0.78	3.2	1.4

[1] DUNNING, J. W., and E. C. LATHROP, Ind Eng Chem., 37 (No. 1), 24 (1945)

Experiments were carried out in a five-cell, glass diffusion battery on a laboratory scale to determine the value of countercurrent extraction of the pentosans from corncob residues. Each of the cells of the battery was

[1] DUNNING, J. W., and L. C. LATHROP, Ind Eng Chem., 37 (No. 1), 24-29 (1945)

equipped with the required inlets and outlets and a heating element. Dilute sulphuric acid at temperatures just under 100°C. was circulated through the crushed corncobs under a variety of conditions.

As a result of these experiments, certain facts become clear. Extraction of 90 to 95 per cent of the pentosans as hydrolyzate products could be accomplished consistently in 80 to 120 min. The hydrolyzates contained 14 to 20 per cent of reducing sugar.

Sharp fractionation of pentosans from hexosans resulted only when conditions were limited. However, high yields of xylose could be obtained under a variety of conditions.

Cellulose Hydrolysis.—The cellulose in the extracted corncobs is hydrolyzed in dilute acid solution after impregnation with concentrated sulphuric acid. One process developed by Dunning and Lathrop was essentially as follows:

The extracted corncobs were dried by filtration; by dewatering devices (to 50 to 55 per cent); and by the use of hot air at temperatures from 60 to 70°C., until the original acid from the pentosan hydrolysis was concentrated to 72 per cent. Residues dried in the presence of acid were more readily saccharified than those freed from acid before drying. The dried material was passed through a hammer mill to reduce it to the desired mesh size.

The dried residue was impregnated with sulphuric acid of 85 per cent concentration by means of a machine constructed of mild steel, which contained a hopper for feeding purposes, a discontinuous screw on a shaft cooled with water, and a water-jacketed casing. Figure 16 shows the effect of variables on the yield of dextrose from extracted corncobs impregnated with 85 per cent sulphuric acid at 40°C. Curve 1 of this figure shows that a 90 per cent yield of dextrose was obtained when 100 lb. of extracted corncobs (of 50-mesh size and with the acid dried into the residue) were treated with slightly under 50 lb. of 85 per cent sulphuric

X = 8-mesh residue, water-washed, and dried.
● = 50-mesh residue, water-washed, and dried.
+ = 50-mesh with acid dried into residue.

Fig. 16.—Effect of variables on yield of dextrose from extracted corncobs impregnated with 85 per cent sulphuric acid at 40°C. [Courtesy of J. W. Dunning and E. C. Lathrop, Ind. Eng. Chem., 37: 24 (1945).]

acid at 40°C. for 2 min. According to the authors, this quantity of sulphuric acid is considerably less than that suggested in some other saccharification processes involving the use of concentrated sulphuric acid.

The residue, impregnated with 85 per cent sulphuric acid, occurring as a dry powder, is diluted with water and permitted to undergo hydrolysis. Dunning and Lathrop have reported that under optimum conditions—

Fig. 17 Flow sheet of the process. [Courtesy of J. B. Dunning and E. C. Lathrop Ind. Eng. Chem., 37: 24 (1945).]

hydrolysis in 8 per cent sulphuric acid for 7 to 10 min. at temperatures of 120 to 130°C.—hydrolyzates were secured that contained 10.5 per cent dextrose.

Flow Sheet of Process.—Figure 17 presents a flow sheet of the process developed by Dunning and Lathrop.[1]

Products.—Table 21 shows the products that may be obtained from 1 ton of agricultural residues.

[1] Dunning, J. W. and E. C. Lathrop, Ind. Eng. Chem., 37: 24 (1945).

TABLE 21.—PRODUCTS OBTAINABLE FROM ONE TON (DRY WEIGHT) OF AGRICULTURAL RESIDUES[1]

Agricultural residue	93 per cent pure crystalline xylose, lb.	Furfural, lb.	95 per cent alcohol, gal.	Lignin, lb.
Corncobs	135	214	44	227
Bagasse.	98	155	49	327
Oat hulls	141	224	42	295
Cottonseed hulls	101	160	42	332
Flax shives . .	111	175	46	525

[1] DUNNING, J. W., and L. C. LATHROP, *Ind. Eng. Chem.*, **37**; 24 (1945).

Uses of the Hydrolyzates.—The dextrose solution obtained by the hydrolysis of cellulose may be used for the production of ethyl alcohol after the lignin has been removed by filtration and the excess acidity has been neutralized with lime.

The xylose solution resultant from the initial (pentosan) hydrolysis of the residues may be used for fermentation, for furfural manufacture, or for the production of crystalline xylose.

Prior to its use for fermentation purposes, it is necessary to neutralize the xylose solution (that obtained by the use of the countercurrent hydrolysis method containing approximately 15 per cent xylose, 0.3 per cent dextrose, 1.75 per cent furfural, 2.0 per cent acetic acid, and 4.4 per cent sulphuric acid) and to remove the furfural. The latter objective may be accomplished, according to Dunning and Lathrop, by feeding the hot xylose solution into a vacuum column and removing the furfural-water azeotrope. The xylose may be fermented to butanol and acetone by *Cl. acetobutylicum* (refer to the chapter on this fermentation) or to 2,3-butanediol by *A. aerogenes* (refer to the chapter concerning this subject).

ACID SACCHARIFICATION OF WOOD

For many years scientists have been engaged in developing and improving methods and equipment for hydrolyzing wood and other cellulose-containing substances to sugars. Their efforts have been rewarded with success, for there are several processes, some fundamentally different, by which sugars may now be prepared on a large scale from such materials.

1. **Historical.**—Dangiville, in 1880, proposed the use of gaseous hydrochloric acid to hydrolyze wood.

In Germany, Classen (1900) developed a method for hydrolyzing vegetable fibers, using sulphur dioxide or sulphuric acid. Attempts to

establish commercial processes in the United States, based on his methods, resulted in failure.

In 1910, Ewen and Tomlinson[1] constructed the first plant for producing ethyl alcohol from sawmill waste in Georgetown, S. C. The average yield of alcohol, using dilute sulphuric acid under high pressure to hydrolyze the sawdust, was approximately 20 gal. per ton, although higher yields were occasionally obtained.

Willstätter (1913) demonstrated that a 40 per cent solution of hydrochloric acid was very much different in action toward cellulose than the common 35 to 36 per cent commercial hydrochloric acid solution. Willstätter, with Zechmeister, discovered that cellulose is transformed without waste to glucose within the space of a few hours by a 40 per cent hydrochloric acid solution at room temperature.

TABLE 22 — PRODUCTION OF ETHANOL FROM CELLULOSE, BY DIFFERENT PROCESSES[a]

Process	Year	Hydrolytic agent	Concentration, per cent	Temperature, degrees Centigrade	Liters of ethanol per 100 kg. of dried wood
Braconnot	1819	H_2SO_4	100		
Arnould	1854	H_2SO_4	100		
Simonsen	1894	H_2SO_4	0 5	180	7 6
Claassen	1900	SO_2	..	150	7 5
Ewen and Tomlinson	1909	SO_2	..	150	8 0
Cohoe	1912	HCl and vapor	.	140	9 0
Willstätter	1913	HCl	40	20	
Hägglund	1914	H_2SO_4	0 4	175	8 8
Kressmann	1915	H_2SO_4	2 5	174	10 7
Meunier	1922	H_2SO_4	.	170	15–20
Scholler	1929	H_2SO_4	0 5	180	22–24
Bergius	1931	HCl	40	20	32–35
Giordani-Leone	1939	H_2SO_4		30	

[a] GIORDANI, M., *Chimica e Industria (Italy)* 21 295 (1939)

Hägglund, Bergius, and others commenced work in 1916 which led to the development of the Rheinau process.

During World War 1, the Germans produced sugars from wood by the so-called "Stettin war process." This process corresponded to the process used by Ewen and Tomlinson in the United States. From August, 1918, to September, 1919, the plant at Stettin[2] produced 300

[1] JACOBS, P. B., and H. P. NEWTON, *U.S Dept. Agr Misc Pub* 327, December, 1938.
[2] SCHOLLER, H., *Chem Zig*, 60: 213 (1936)

tons of sugar and converted it to **150,000 liters** of ethyl alcohol. Owing to the low yields, only about 6 liters of alcohol per 100 kg. of dry wood substance, it was economically unsound to continue operation of the plant after the war was over. It was later shown that the poor yields had been due to a partial destruction of the sugars during the hydrolytic process.

At Geneva, Terrisse and Lévy in 1920 used a combination of 40 per cent hydrochloric acid solution and gaseous hydrochloric acid to hydrolyze wood in a method that later became known as the Prodor process.[1]

Much research concerning the production of ethyl alcohol from wood wastes has been carried out by the U S. Forest Products Laboratory at Madison, Wis. Sherrard and Kressman[2] reviewed the development of wood saccharification processes in the United States from the time of their inception up to prior to World War II. Harris, Beglinger, Hajny, and Sherrard[3] studied the hydrolysis of wood with dilute sulphuric acid in a stationary digester. They found that yields of about 50 per cent of reducing sugar (75 to 80 per cent fermentable) could be obtained from softwoods; and of about 50 per cent of reducing sugar (50 to 65 per cent fermentable) from hardwoods

The use of the rotary digester in wood saccharification was investigated by Plow, Saeman, Turner, and Sherrard.[4] They reported that there were some serious disadvantages and little advantage in the use of the rotary digester in multistage saccharification of wood as compared with the vertical stationary percolator.

Saeman[5] studied the kinetics of the hydrolysis of wood and of the decomposition of sugars in dilute acid at high temperatures. He found that the hydrolysis of hemicellulose took place easily and required only a fraction of the time needed for the hydrolysis of the resistant portion of cellulose. Saccharification of cellulose involved two reactions: the hydrolysis to reducing sugar and the consecutive decomposition of the sugars. The decomposition of all the sugars appeared to follow the laws of a first-order reaction An increase in acid concentration (0 4 to 1 6) or temperature (170 to 190°C) resulted in increased efficiency in the formation of reducing sugar from cellulose.

An analytical procedure, stated to be rapid, was developed for the quantitative saccharification of wood and cellulose by Saeman, Bubl, and

[1] ORMANDY, W. R., *Jour Soc. Chem Ind (Trans)*, **45**: 267T (1926)

[2] SHERRARD, E C , and F. W. KRESSMAN, FPL Report No R1454, Madison, Wis , September, 1944.

[3] HARRIS, E. E, E. BEGLINGER, G. J HAJNY, and E. C. SHERRARD, *Ind. Eng. Chem.*, **37** (No. 1) 12 (1945)

[4] PLOW, R H., J F SAEMAN, H D. TURNER, and E. C. SHERRARD, *Ind. Eng. Chem.*, **37** (No 1). 36 (1945)

[5] SAEMAN, J F., *Ind Eng Chem*, **37** (No 1): 43 (1945).

Harris.[1] The material for analysis is ground sufficiently finely to pass through a 30-mesh screen. It is treated with 72 per cent sulphuric acid at 30°C. for 45 min. after air-drying to a low moisture content. The mixture is diluted with water and treated for 1 hr. at a steam pressure of 15 lb., or by boiling for 4.5 hr.

Procedures for analyzing wood sugars have been described by Saeman, Harris, and Kline.[2] These included an electrometric titration method and a micro method developed by Shaffer and Somogyi for determining the reducing sugars; and a test for fermentable sugars.

Table 22 on page 99, summarizes some data concerning different methods for producing ethanol from cellulose.

2. The Bergius-Rheinau Process.—The Bergius[3] process is based on Willstätter's discovery that a 40 per cent solution of hydrochloric acid will hydrolyze cellulose to glucose at room temperature.

Cellulose is the principal constituent of wood. Hence a 40 per cent hydrochloric acid solution dissolves most of the wood except the lignin

In the Bergius process, the wood is shredded and then dried in revolving drums to a water content of approximately 0.5 per cent. The dried wood is next conveyed to a battery of diffusers, where hydrochloric acid acts upon it to convert it to water-soluble sugars and other end products. A countercurrent principle is employed in the extraction. The acid progresses through the battery (Fig. 18) and comes into contact with fresh wood in the first diffuser. During the extraction process, about two-thirds of the wood by weight is dissolved, while one-third remains as lignin. The resultant acid solution contains approximately 32 per cent by volume of reducing sugar[4]

The acid solution containing the reducing sugars (the hydrolyzate) is now distilled at 36°C. under vacuum in order to separate the bulk of the acid from the sugar. This procedure removes about 80 per cent of the acid from the hydrolyzate. The acid is regenerated, reconcentrated, and used over again.

The hydrolyzate is further concentrated in a spray-drying chamber where water and hydrochloric acid are lost by evaporation. The resultant solid product, which is voluminous, is collected in a cyclone, an apparatus that removes particles of dried material from the air by centrifugal force

[1] SAEMAN, J. F., J. L. BUHL, and L. F. HARRIS. Ind. Eng. Chem., Anal. Ed., 17 (No. 1): 35 (1945).
[2] SAEMAN, J. F., L. F. HARRIS, and A. A. KLINE. Ind. Eng. Chem., Anal. Ed., 17 (No. 2): 95 (1945).
[3] BERGIUS, F. Ind. Eng. Chem., 29: 247 (1937).
[4] Ibid.
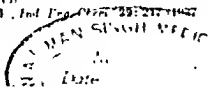
There are approximately **90 per cent** sugars, 8 per cent water, and 1 to 2 per cent of hydrochloric acid in the solid hydrolyzate.

The sugars of the hydrolyzate, which are usually glucose, fructose, mannose, galactose, and xylose (depending on the nature of the wood hydrolyzed), are principally in a tetrameric form and are not directly fermentable. However, **by diluting the solid hydrolyzate with 3 parts by volume of water and by heating the resulting solution at 120°C. for**

1. Wood supply
2. Wood shredder
3. Chips
4. Wood dryer
Disintegration steps:
5. Hydrochloric acid
6. Dried wood chips
7. Diffusion battery
8. Lignin

Spraying steps
15. Air heater
16. Spray dryer
17. Cyclone separator
18. Crude sugars (raw product)
Crystallization steps:
19. Vacuum pump
20. Inversion
21. First crystallization
22. First crystallization
23. Filter press
24. Solution vat
25. Recrystallization
26. Centrifuge for pure crystals
27. Sirup filtrate (mother liquor)
28. To alcohol (by fermentation)

13. Hydrochloric acid
14. Sirup

Fig. 18—Flow diagram of the Bergius process. (*Courtesy of Dr. F. Bergius, Ind. Eng. Chem.*, **29**: 247 (1937).)

0.5 hr., the tetrameric form is converted to a fermentable form. Galactose and xylose are not fermentable by yeast.

According to Bergius, approximately 80 per cent of the raw sugar obtained by hydrolysis is fermented to alcohol, a long ton of dry wood yielding 85 to 90 gal. of ethyl alcohol (190 proof). The other 20 per cent of the sugar may be recovered and used for fodder or for other purposes.

The lignin, washed free of acid, may be used as fuel, or pure charcoal may be prepared from it.

Acetic acid, in quantities that correspond to those recovered during the destructive distillation of wood, is obtained as a by-product of this process.

3. The Scholler-Tornesch Process.[1]—In contrast to the Bergius process, the Scholler process employs dilute acid, elevated temperatures, and steam under pressure. The wood used does not have to be dried or of any definite particle size.[1] Furthermore, no attempt is made to recover the acid.

In 1926 and 1927 Scholler carried out research in the laboratory that indicated the value of the periodic removal of the sugar formed by pressure percolation. In Fig. 19 the curve X represents theoretical saccharification; the curve Z_1, the course of saccharification by the percolation method; and curve Z, the course of saccharification by a method that corresponds to the Stettin war process.
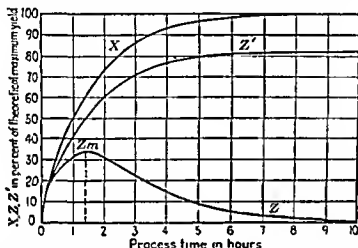
Fig. 19—Graphical representation of course of saccharification in enclosed autoclave and percolator *(Courtesy of Dr. H. Scholler, Chem. Ztg., 60, 293 (1936).)*

In the present industrial process, comminuted wood is carried by a system of conveyers to the top of a battery of percolators into which it is packed. Dilute acid solution is permitted to percolate intermittently under pressure through the heated wood, the accumulating sugar solution being removed periodically to prevent it from becoming destroyed. The sugar solution is neutralized with $CaCO_3$, permitted to settle in a tank, and filtered. It may then be fermented by yeast.

As stated above, the particle size of the wood is not of great importance. Sawdust, plane dust, rasp dust, and similar types of wood of variable moisture content may be used directly.

Each percolator is 14 m. in length and 2.1 m. in diameter with a capacity for 50 cu. m. of material. It is filled loosely with the cellulose-containing substance. After the percolator is closed, steam pressure is applied suddenly from above upon the loosely packed material, with the

result that it is compressed evenly. The percolator is filled again and the material again compressed. The procedure is repeated until the percolator is almost completely filled with the compressed cellulose material.

The dilute acid solution used for hydrolysis is prepared by pumping sulphuric acid into water, which has previously been warmed by passage through a heat-exchange apparatus, to give an acid concentration of approximately 0.4 per cent.

This dilute acid flows through the cellulose-containing material at a temperature of about 170°C. and a pressure of 8 atmospheres. The sugar solution thus formed is removed shortly, passing through the heat exchanger where it gives off heat to water being warmed for the dilute acid solution. Hydrolytic action continues in the percolator after the removal of the sugar solution, owing to the moisture, acid, heat (160 to 190°C), and pressure, but no additional sugar is removed until the next passage of the hydrolyzing solution.

After the sugars have been recovered from the wood waste by intermittent percolation with dilute acid, the lignin residue, which is in the form of a hard cake, must be removed from the percolator. Since lignin has a high water content and is at a temperature of about 180°C. at the end of the process, the sudden release of a section in the lower portion of the percolator causes an explosive expansion of the water in the lignin and the cake is shattered

From the sugars obtained by the preceding method, industrially important alcohols, acids, or yeast may be prepared.

Ethyl alcohol yields amounting to 60 to 70 per cent of the theoretical are obtained by this process.

Lignin, acetic acid, furfural, and waste sugars are by-products.

TABLE 23 —PRODUCTS OBTAINED BY DIFFERENT PROCESSES FROM 100 KG. OF DRIED WOOD[1]

	Bergius, kg.	Scholler, kg.	Giordani-Leone, kg
Acetic acid	4	[2]	0 2
Furfural		[2]	2
Lignin	33	30	30
Total sugars	66	43 3	60
Sugars fermented by yeast	56	35 2	48
Sugars not fermentable by yeast[3]	10	8 1	12
Yield of ethanol (liters of 100 per cent)	35	22	30

[1] Prepared from data presented by M. Giordani *Chimica e industria (Italy),* 21:265–272 (1939)
[2] Data not available
[3] Approximate figures   May be utilized by bacteria in production of butanol, etc

4. The Giordani-Leone Process.—This process, described and illustrated by Giordani,[1] makes use of 60°Bé. sulphuric acid. Space does not permit a detailed account of this process but some data concerning this, the Bergius, and the Scholler processes are presented in Table 23,

1 Elevator
2 Autoclave
3 H₂SO₄ per cent) vat
4 Press
5 Dryer
6 Mash
7 Furfural column
8 Pulping apparatus
9 Hydrolysis vat
10 Lignin filter
11 Neutralization vat
12 Calcium sulphate filter
13 Fermentation tank
14 Distillation column

A Pipe system of H₂SO₄ at 60° Bd.
B Steam-pipe system
C Water-pipe system
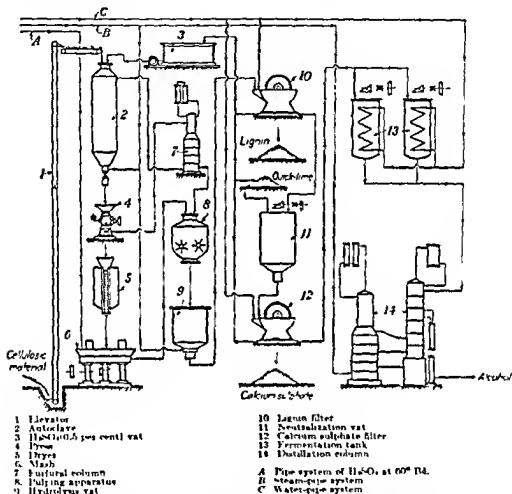
Fig. 20 Scheme of Giordani-Leone process for the saccharification of cellulosic materials
[Courtesy of Dr M Giordani Chimica e industria (Italy) 21 265 (1939)]

on page 104 Of the sugar derived from wood by the Giordani-Leone process, that part which is unfermentable by yeast may be used by *Clostridium acetobutylicum* for the production of butanol, acetone, and ethanol The efficiency of the Giordani-Leone process is said to be high

Figure 20 shows a scheme of the Giordani-Leone process for the saccharification of cellulosic materials

[1] GIORDANI, M., *Chimica e industria* (Italy), **21**: 265–272 (1939)

FIG 21.—Equipment for saccharification of wood and fermentation of wood-sugar solutions. (*Courtesy of E. E. Harris and E. Beglinger, Mimeo Report No. R-1617, Forest Products Laboratory, U. S. Dept Agr , Madison, Wisconsin, June, 1946.*)

5. The Madison Wood-Sugar Process.[1]—This process was developed by the Forest Products Laboratory of the U.S. Department of Agriculture at Madison, Wis. Wood waste, consisting of edgings, chipped slabs, shavings, and sawdust, is hydrolyzed by permitting to flow continuously through it dilute sulphuric acid at temperatures of 150 to 185°C. Furfural and methanol are separated from the hydrolyzate by flashing. The hydrolyzate is then automatically neutralized with lime and filtered while under pressure. The solution of wood sugars may be used for purposes

FIG. 22 — Equipment for neutralization of wood sugars.   (Courtesy of E. E. Harris and E. Beglinger, Mimeo Report No R-1617, Forest Products Laboratory, U S Dept Agr, Madison, Wis, June, 1946.)

such as the production of ethyl alcohol by fermentation with *Saccharomyces cerevisiae*.

The pilot-plant equipment for hydrolyzing wood wastes is shown in Figs. 21 and 22. It consists essentially of the following elements: a hog chipper used to break up pieces of wood waste to the desired size, facilities for lifting the waste to the storage chamber which is located directly above the digester; a digester constructed of silicon bronze having a capacity of 27 cu ft, and of 23-in inside diameter, with a reducing cone at the top and another plus n screen at the bottom; a water pump with a capacity variable from 2 to 10 gal per min ; an acid pump with a capacity variable from 0.2 to 1 gal per hr ; n switch for controlling the two pumps; a water heater with facilities for introducing steam into the water; n jet located in the water line for adding acid, two inlets at the top of the digester for introducing the dilute acid, a flash tank; receiving tanks with mixers; a neutralizing kettle, storage tanks; and n receiver for the lignin

The wood waste used, Douglas fir, produces best results when present

[1] HARRIS, E. E. and E. BEGLINGER, Report No R-1617, Forest Products Laboratory, Madison, Wis, June, 1946, also Ind Eng Chem, 38 (No 9) 890 (1946)

Fig. 21.—Equipment for saccharification of wood and fermentation of wood-sugar solutions. (*Courtesy of E. E. Harris and E. Beglinger, Mimeo Report No. R-1617, Forest Products Laboratory, U. S. Dept. Agr., Madison, Wisconsin, June, 1946*)

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Fig. 22—Equipment for neutralization of wood sugars   (*Courtesy of E E Harris and E Beglinger, Mimeo. Report No R-1617, Forest Products Laboratory, U S Dept Agr, Madison, Wis, June, 1916*)

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The pilot-plant equipment for hydrolyzing wood wastes is shown in Figs. 21 and 22. It consists essentially of the following elements: a hog chipper used to break up pieces of wood waste to the desired size, facilities for lifting the waste to the storage chamber which is located directly above the digester; a digester constructed of silicon bronze having a capacity of 27 cu. ft. and of 23-in. inside diameter, with a reducing cone at the top and another plus a screen at the bottom; a water pump with a capacity variable from 2 to 10 gal. per min.; an acid pump with a capacity variable from 0.2 to 4 gal. per hr.; a switch for controlling the two pumps, a water heater with facilities for introducing steam into the water, a jet located in the water line for adding acid; two inlets at the top of the digester for introducing the dilute acid; a flash tank; receiving tanks with mixers; a neutralizing kettle; storage tanks; and a receiver for the lignin

The wood waste used, Douglas fir, produces best results when present

in the following percentages: 25 to 30 per cent shavings, 25 to 30 per cent sawdust, and 40 to 50 per cent edgings and slabs. These are hogged in such a manner that the pieces vary from 0.25 to 1 in. in length. Green wood waste containing 30 to 50 per cent of moisture is more satisfactory than dry wood because of the greater ease with which the acid diffuses through the material. Bark usually constitutes 35 to 50 per cent of the waste used. Yields of ethyl alcohol, used as an index of the efficiency of hydrolysis, are calculated on the basis of dry, bark-free wood. From 2 to 2.5 tons of green, bark-containing wood waste are generally necessary to yield a ton of bark-free wood on the dry basis.

During hydrolysis, best results are obtained when the digester is packed evenly with a uniform mixture of the hogged shavings, sawdust, edgings, and slabs. The digester is filled with the wood waste, the cover is put in place, and steam (at a pressure of 150 lb. per sq. in.) is introduced rapidly at the top (with vents open at the bottom of the digester) until the gauge at the top of the digester records a pressure of 10 lb. The steam is then turned off. When the gauge pressure has dropped to zero, the cover is opened and a new charge introduced, followed again by steam packing. After the packing is completed, steam is introduced through the bottom, with the top vents open, to remove more air and to continue the heating of the wood waste. Finally, the vents are closed and steam introduced to yield a pressure of 50 lb. in the digester.

Acid is introduced into the digester as follows: At the beginning, 200 lb. of sulphuric acid at a concentration of 1.5 to 2.0 per cent and at a temperature of 150°C. are added rapidly at the top of the digester. This amount of acid is required to bring the average acid concentration of the moisture present in the wood waste and added by the steam (about 600 lb. in all) to 0 5 to 0.6 per cent. Next, sufficient sulphuric acid (at a concentration of 0.5 to 0.6 per cent and at a temperature of 150°C.) is added at the rate of 12 gal. per min. to bring the ratio of dilute acid to wood (dry basis) to 3 to 1. This usually amounts to 400 to 600 lb. of dilute acid. Finally, 30 min. after the first acid has been added, the acid pump is started and dilute acid at a concentration of 0.5 to 0.6 per cent is added at the rate of 20 lb. per min. At the same time a temperature controller is set to cause the temperature in the digester to increase by 0.5°C. per min. for 30 min., or until a final temperature of 185°C. is obtained. This temperature is maintained until the end of the run.

Simultaneously with the addition of the dilute acid from the pump, the acid-sugar solution is removed continuously from the bottom of the digester at a rate of about 20 lb. per min. Thus the rate of addition of the acid f ͭ ͪᵉ digester and that of the removal of the hydrolyzate are the sa

The hydrolysis is continued until the average reducing-sugar concentration of the total combined hydrolyzate is 5.0 per cent This corresponds to a discontinuation of the hydrolysis after the concentration of reducing sugar in the hydrolyzate has dropped to 0 5 per cent or less.

The hydrolyzate is passed to a flash chamber where the pressure is dropped to 30 lb. per sq. in, permitting the steam to carry off the methanol and furfural. The latter are passed through a heat exchanger and, while being cooled, heat the water that is used in making the dilute acid.

The sugar solution is automatically neutralized with lime and then filtered to remove calcium sulphate while still at a pressure of 30 lb

Finally, the sugar solution is cooled to 30°C. by flashing, and the precipitate which settles out is removed by filtration.

The usual time required for hydrolysis is 2 6 to 3 0 hr, although occasionally it may be slightly shorter or longer

A ton of dry, bark-free Douglas fir wood waste, when hydrolyzed by the foregoing process, yields 61 5 gal. of 95 per cent ethyl alcohol.

The advantages of this process as compared with the Scholler process are: the shorter time required for hydrolysis, the lower steam requirement, the rapid removal of the sugars from the digester and the neutralization of the acid, which prevents their decomposition; the production of fewer substances inhibitory to fermentation; the longer life of the valves and other equipment, on account of fewer changes during operation; and the higher yields of sugars and of alcohol from the hydrolyzed wood waste.

References on Saccharifying Agents

Adams, S. L., B. Balankura, A. A. Andrasen, and W. H. Stark: Submerged Culture of Fungal Amylase, *Ind Eng Chem*, 39: 1615–1617 (1947)

Anderson, J. Ansel: "Enzymes and Their Role in Wheat Technology," Interscience Publishers, Inc., New York, 1946

Anonymous: List of Publications on Chemistry of Wood and Derived Products, *Report No R238, U S D A, Forest Service, Forest Products Laboratory*, Madison, Wis, December, 1945

Balankura, B., F. D. Stewart, R. E. Scalf, and L. A. Smith: Submerged Culture of Molds for Amylase Production, *Jour Bact*, 61: 591 (1946)

Berkesund, H., and L. M. Christensen: The Production of Ethyl Alcohol from Cull Potatoes and Other Farm Crops, *Bull No 211, Univ Idaho Agr Expt Sta*, Moscow, Idaho, 1941

Bergius, F.: Conversion of Wood to Carbohydrates and Problems in the Industrial Use of Concentrated Hydrochloric Acid, *Ind Eng Chem*, 29: 247 (1937)

Boyd, J W, and L. A. Underkofler: Mold Bran Aids Production of Grain Alcohol, *Chem & Met Eng*, 52 (No 12): 110–111 (1945)

Christensen, L. M.: Method of Promoting Mold Growth, U S Patent 2,312,168, 1911

Classen, H.: Die Wirtschaftlichkeit der Herstellung von Holzzucker, *Chem Ztg*, 56: 689 (1932)

CORMAN, J , and A. F. LANGLYKKE: Action of Mold Enzymes in Starch Saccharification, *Cereal Chem.*, 25 (No. 3): 190–201 (1948).

DUNNING, J. W., and E. C. LATHROP: The Saccharification of Agricultural Residues, *Ind Eng. Chem.*, 37 (No. 1): 24–29 (1945).

EFRON, A., and R. H. BLOW: Production of Maltose Sirups from Granular Wheat Flour, *Ind. Eng Chem*, 40 (No. 3). 412–415 (1948).

ERB, N. M., and F. M HILDEBRANDT: Mold as an Adjunct to Malt in Grain Fermentation, *Ind. Eng Chem.*, 38 (No. 8): 792–794 (1946).

————, R. T. WISTHOFF, and W. L. JACOBS: Factors Affecting the Production of Amylase by *Aspergillus niger*, Strain NRRL 337, when Grown in Submerged Culture, *Jour. Bact*, 55 (No. 6): 813–821 (1948).

FOTH, G : "Handbuch der Spiritusfabrikation," Paul Parey, Berlin, 1929.

GATES, R. L , and E KNEEN. Preparation of Amylase-active Concentrates from Mold Bran, *Cereal Chem*, 25 (No. 1): 1–19 (1948).

GIORDANI, M : Alcool da materiali cellulosici, *Chimica e industria (Italy)*, 21: 265–272 (1939)

GROVE, O . The Amylo Process of Fermentation, *Jour. Inst. Brewing*, 20: 248–261 (1914).

HAO, L C., E I FULMER, and L. A. UNDERKOFLER: Fungal Amylases as Saccharifying Agents in the Alcoholic Fermentation of Corn, *Ind Eng. Chem.*, 35: 814–818 (1943).

————, and J A JUMP: Microbial Amylase Preparations—Conversion Agents for Alcoholic Fermentation, *Ind. Eng Chem*, 37: 521–525 (1945).

HARRIS, E E Saccharification of Wood, *Report No R1475, Forest Products Laboratory*, Madison, Wis , March, 1945.

————, and E. BEGLINGER: Madison Wood-sugar Process, *Ind. Eng. Chem* , 38 (No. 9). 890–895 (1946)

————, G J HAJNY, and E C SHERRARD· Hydrolysis of Wood. Treatment with Sulfuric Acid in a Stationary Digester, *Ind. Eng. Chem.*, 37 (No 1): 12–23 (1945).

HAWLEY, L F , and L E. WISE: "The Chemistry of Wood," Rheinhold Publishing Corporation, New York, 1926.

HAYEK, M , and R L SHRINER Hydrolysis of Starch by Sulfurous Acid, *Ind. Eng. Chem.*, 36 (No. 11): 1001–1003 (1944).

JEFFREYS, G A : Mold Enzymes, Produced by Continuous Tray Method, *Food Inds* , 20: 688–690, 825, 826 (1948)

KATZEN, R , and D F. OTHMER, Wood Hydrolysis—A Continuous Process, *Ind. Eng. Chem* , 34 (No 3). 314–322 (1942).

KRESSMAN, F. W.: The Manufacture of Ethyl Alcohol from Wood Waste, *U S Dept Agr , Misc Bull* 983, 1922.

LE MENSE, E H , J. CORMAN, J. M. VAN LANEN, and A. F. LANGLYKKE: The Production of Mold Amylases in Submerged Culture, *Jour. Bact* , 54 (No 2): 149–159 (1947).

————, V. E SOHNS, J. CORMAN, R. H. BLOW, J. M VAN LANEN, and A F. LANGLYKKE: Grain Alcohol Fermentations, Submerged Mold Amylase as a Saccharifying Agent, *Ind Eng Chem*, 41 (No 1): 100–103 (1949)

LOCKE, E G , J F SAEMAN, and G. K. DICKERMAN: The Production of Wood Sugar in Germany and Its Conversion to Yeast and Alcohol, *Fiat Final Report*, No 499, Nov. 14, 1945

OLSEN, W. J., R. EVANS, and A. D. DICKSON: A Modification of the Kneen and Sandstedt Methods for the Determination of Alpha- and Beta- Amylases in Barley Malt, *Cereal Chem.* 21: 533–539 (1944).

———, ———, and ———: A Corrected Unit of Alpha-Amylase Activity, *Cereal Chem.*, 24: 299–300 (1947).

ORMANDY, W. R.: Sugar from Wood, *Jour. Chem. Ind.* (*Trans.*), 45: 267T (1926).

ORTVED, W. C.: The Application of *Taka-koji* in Distilleries, *Orig. Com. 8th Int. Cong. Appl. Chem.*, 26: 69–76 (1912).

OWEN, W. L.: Production of Industrial Alcohol from Grain by the Amylo Process, *Ind. Eng. Chem.*, 25: 87–89 (1933).

PLOW, R. H., J. F. SAFMAN, H. D. TURNER, and E. C. SHERRARD: The Rotary Digester in Wood Saccharification, *Ind. Eng. Chem.*, 37 (No. 1): 36–43 (1945).

ROBERTS, M., S. LAUFER, D. STEWART, and I. T. SALETAN: Saccharification of Wheat by Fungal Amylases for Alcohol Production, *Ind. Eng. Chem.*, 36: 811–812 (1944).

ROSE, D.: The Amylase of *Bacillus polymyxa*, *Arch. Biochem.*, 16 (No. 3): 349–355 (1948).

SAFMAN, J. F.: Kinetics of Wood Saccharification—Hydrolysis of Cellulose and Decomposition of Sugars in Dilute Acid at High Temperatures, *Ind. Eng. Chem.*, 37 (No. 1), 43–52 (1945).

———, J. L. BUHL, and F. E. HARRIS: Quantitative Saccharification of Wood and Cellulose, *Ind. Eng. Chem.*, *Anal. Ed.*, 17 (No. 1), 35–37 (1945).

———, E. E. HARRIS, and A. A. KLINE: Analysis of Wood Sugars, *Ind. Eng. Chem.*, *Anal. Ed.*, 17 (No. 2), 95–99 (1945).

SANDSTEDT, H. M., E. KNEEN, and M. J. BLISH: A Standardized Wohlgemuth Procedure for Alpha-Amylase Activity, *Cereal Chem.*, 16: 712–723 (1939).

SCHOENE, L., E. I. FULMER, and L. A. UNDERKOFLER: Saccharification of Starchy Grain Mashes for the Alcoholic Fermentation Industry, Comparison of General Saccharifying Agents, *Ind. Eng. Chem.*, 32: 544–547 (1940).

SCHOLLER, H.: Die Gewinnung von Zucker, Spiritus und Futterhefe aus Holz als Rohstoff, *Chem. Ztg.* 60: 293 (1936).

———: French Patents 706678, 777824, and 799358, U.S. Patents 1,641,771, 1,890,-301; 1,990,097; 2,083,317, 2,083,318, 2,086,063 2,088,977, 2,108,567, 2,123,211, 2,123,212, 2,188,192, and 2,188,193

STEVENSON, G. M., and K. J. GORAING: Saccharification of Grain Mashes for the Alcoholic Fermentation Industry. Plant-scale Use of Mold Amylase, *Ind. Eng. Chem.*, 38: 980–985 (1946).

SHERRARD, E. C., and F. W. KRESSMAN: A Review of Wood Saccharification Processes in the United States Prior to World War II, *Report No. R1451, Forest Products Laboratory*, Madison, Wis., September, 1944.

SOMOGYI, M.: A New Reagent for the Determination of Sugars, *Jour. Biol. Chem.*, 160: 61–68 (1945).

STARK, W. H., S. L. ADAMS, R. E. SCALF, and P. KOLACHOV: Laboratory Cooking, Mashing, and Fermentation Procedures, *Ind. Eng. Chem.*, *Anal. Ed.*, 15: 413–416 (1943).

TAKAMINE, J.: Process for Producing Diastatic Product, U.S. Patent 1,054,324, 1913.

———: Diastatic Product, U.S. Patent 1,054,626, 1913.

———: Enzymes of *Aspergillus oryzae* and the Application of Its Amylolytic Enzyme to the Fermentation Industry, *Ind. Eng. Chem.*, 6: 824–828 (1914).

————: Diastatic Product and the Process for Producing the Same, U.S. Patent 1,148,938, 1915.

————: Process for Producing Diastatic Product, U.S. Patent 1,263,817, 1918.

THORNE, C B., R. L. EMERSON, W. J. OLSON, and W. H. PETERSON: Evaluation of Malts for Production of Alcohol from Wheat, *Ind. Eng. Chem.*, 37: 1142–1144 (1945).

UNDERKOFLER, L. A : Production of Diastatic Material, U.S Patent 2,291,009, 1942

————: Microbial Amylases Their Application to Alcoholic Fermentation, *Brewers Digest*, 17 (No. 11): 29 (1942).

———— and E I FULMER: *Chronica Botan.*, 7: 420 (1943).

————, ———— and L SCHOENE: Saccharification of Starchy Grain Mashes for the Alcoholic Fermentation Industry   Use of Mold Amylase, *Ind Eng Chem*, 31: 734–738 (1939)

————, G. M. SEVERSON, and K. J. GOERING. Saccharification of Grain Mashes for Alcoholic Fermentation, *Ind Eng Chem.*, 38: 980–985 (1946).

————, ————, ———— and L M. CHRISTENSEN: Commercial Production and Use of Mold Bran, *Cereal Chem*, 24 (No 1): 1–22 (1947).

UNGER, E D., H. I' WILLKIE, and H C. BLANKMEYER: The Development and Design of a Continuous Cooking and Mashing System for Cereal Grains, *Trans. Am Inst Chem Engrs.*, 40: 421–443 (1944).

VAN LANEN, J M , and R H BLOM: "Report on Production and Utilization of Mold Bran," Northern Regional Research Laboratory, Peoria, 1945

———— and E H LE MENSE: The Production of Fungal Amylases in Submerged Culture and Their Use in the Production of Industrial Alcohol, *Jour. Bact.*, 51: 595 (1946).

VOGEL, E H , F. H. SCHWAIGER, H G. LEONHARDT, and J. A. MERTEN: "The Practical Brewer," Master Brewers Association of America, St. Louis, Mo , 1947

WILLKIE, H F : Distillery Processes, *Chem. & Met. Eng.*, May, 1945.

———— and P J KOLACHOV: "Food for Thought," Indiana Farm Bureau, Inc., Indianapolis, 1942.

WOHLGEMUTH, J : Über eine neue Methode zur quantitativen Bestimmung des diastatischen Ferments, *Biochem. Zeit.*, 9: 1–9 (1908).

CHAPTER IV

THE PRODUCTION OF INDUSTRIAL ALCOHOL
BY FERMENTATION

One of the most important and best-known industrial fermentations is that in which ethyl alcohol is produced from sugars by yeasts. The chemical manufacturer, the brewer, the distiller, the baker, the vinegar manufacturer, the scientist, the housewife, and many others depend in one way or another on the ability of the yeast to convert sugars to alcohol, carbon dioxide, and other end products. Since low-priced and waste carbohydrate materials may be used in the manufacture of ethyl alcohol, the fermentation process has not only great present but also vast potential value.

The subject of ethyl alcohol production by fermentation has assumed new interest on account of attempts to find substitutes for gasoline Blends of alcohol with gasoline, especially a blend containing 10 per cent ethyl alcohol, may be used satisfactorily in the present internal-combustion type of motor. Present-day demands for motor fuels are great, but the sources of petroleum are limited

Definitions.—Ethyl alcohol (CH_3CH_2OH), or ethanol, may be referred to by other names For example, the alcohol may be named to indicate the source of raw material from which it is manufactured or to indicate the general purpose for which it is to be used Grain alcohols are alcohols made from grains, such as corn, wheat, or rice. The term "grain alcohol" is sometimes used to signify ethyl alcohol in contrast to methyl alcohol (CH_3OH), or methanol, which is manufactured by the destructive distillation of wood, by synthesis, or by other means Molasses alcohol is alcohol produced from sugar-cane molasses Industrial alcohol is ethyl alcohol used for industrial purposes Under this term is included power, or fuel alcohol, i e , alcohol used in combination with gasoline or other motor fuels.

The units of measurement commonly used in reporting ethyl alcohol production are the proof gallon, wine gallon, tax gallon, and barrel The standard proof gallon is a wine gallon containing 50 per cent by volume (100 proof) of ethyl alcohol A wine gallon is equal to a standard United States gallon and contains 231 cu in A tax gallon is equivalent to a proof gallon for spirits of a concentration of 100 proof or more and is

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equivalent to a wine gallon for spirits of a concentration less than 100 proof. A barrel contains 31 wine gallons.

Proof is twice the per cent in volume of ethyl alcohol. For example, 95 per cent ethyl alcohol by volume is 190 proof.

Production Statistics.—The quantity of ethyl alcohol produced by 11 leading states and by the United States during the fiscal years 1942-1946 is shown in Table 24.

TABLE 24.—ETHYL ALCOHOL PRODUCTION BY LEADING STATES DURING FISCAL YEARS 1942–1946[1,2]

(Production, proof gallons)

State	1942	1943	1944	1945	1946
Pennsylvania	101,768,211	84,702,184	146,992,133	165,501,630	62,641,608
Louisiana	89,499,307	106,675,571	120,559,576	140,248,183	73,155,800
West Virginia	50,147,368	50,555,545	43,461,248	43,304,054	45,428,545
New Jersey	46,417,013	31,096,102	52,446,671	50,054,772	22,620,682
Indiana	39,841,119	30,538,951	53,342,979	28,242,636	28,078,096
Maryland	35,030,299	39,709,182	49,069,064	32,510,956	24,509,586
Texas	17,357,641	20,409,056	22,999,430	20,623,120	25,596,244
Kentucky	2,421,565	21,617,016	35,555,383	22,527,183	379,884
Nebraska			4,265,932	40,933,582	22,988,357
Iowa		1,509,427	11,337,349	27,779,233	7,827,807
California	8,577,061	12,191,672	16,939,304	10,612,619	7,828,146
Total (United States, including Hawaii and Puerto Rico)	424,804,091	447,786,568	636,575,216	683,431,544	353,524,384

[1] U S Treas Dept, Annual Report of the Commissioner of Internal Revenue, 1943, 1944, 1945, and 1946

[2] Includes production by redistillation

Raw Materials.—Ethyl alcohol may be produced from any fermentable sugar by yeasts under suitable conditions. Since starches and certain other carbohydrates may be hydrolyzed to fermentable sugars by biological or chemical means, there are available many possible sources of sugar.

1. Types —Raw materials may be classified into three principal types: (a) the saccharine materials, such as sugar cane, sugar beets, molasses, and fruit juices; (b) the starchy materials, which include the cereals (corn, malt, barley, oats, rye, wheat, rice, grain, sorghum, and the like), potatoes, sweet potatoes, Jerusalem artichokes (girasol), manioca meal, and other substances; and (c) cellulosic materials, such as wood and waste sulphite liquor.

KS

PUMPING MOLASSES
TO TANK STEAMER

TRANSPORTING
MOLASSES TO U S A

CORN STORAGE
ELEVATORS

TRANSPORTING
CORN TO HAMBURG-MOLE

ENDING MILL

CORN
STORAGE ELEVATOR

STORAGE
KS

PUMPING MOLASSES
TO STORAGE TANKS

PURE ALCOHOL FOR
THE RHISH OLEIN
PERMIT REQUIRED

NITROUS COMPOUNDS
AND IMPURITIES

PHARMACEUTICAL
USES

PURE EXTRACTS
AND FLAVORINGS

FOR
UNITED
STATES

PURE ALCOHOL
PERMIT REQUIRED

2. *Principal Raw Materials Utilized.*—In the United States, molasses and grain are the principal carbohydrate materials used in the production of ethyl alcohol. In Table 25 may be found the raw materials used for ethyl alcohol production during the fiscal years 1939, and 1941 to 1947.

Various types of raw materials are used in different countries. In Germany, for example, potatoes are used extensively, in France, sugar beets. Sweden manufactures much industrial alcohol from sulphite pulp. In Italy, sugar beets, molasses, grapes, and other substances are used.

PROCESSES OF MANUFACTURE

The process used in the manufacture of ethyl alcohol by fermentation depends on the nature of the raw material. Saccharine materials usually require little or no special preliminary treatment other than dilution and may be fermented directly after certain customary adjustments have been made in the mash. But starchy and cellulosic materials must be hydrolyzed to fermentable sugars before they can be utilized by the yeast.

In each of the processes, success depends on the efficiency of preliminary treatment, if any; the use of an optimum concentration of sugar, an optimum pH, and optimum temperature, the addition of nutrient substances to the mash, if it be deficient in any essential constituent, inhibition of bacterial growth; the use of a vigorous strain of yeast with high alcoholic tolerance and so capable of producing large yields of alcohol; the maintenance of anaerobic conditions during the fermentation proper; and the prompt distillation of the fermented mash.

1. *Ethyl Alcohol from Molasses.* a. *Outline of Process.*—The molasses mash is adjusted to the desired sugar concentration and temperature by the addition of water and to the desired pH by the addition of a measured quantity of acid. A yeast "starter" is mixed with the mash in the fermentation tank, which is usually covered, in one of several manners. Streams of the adjusted mash and the starter flowing simultaneously into the fermenter may be caused to converge on a baffle board located in the upper part of the tank. The mash and starter become well mixed as they spatter and fall to the bottom of the tank. An alternate method is to add the starter after the mash has been placed in the tank and effect mixing of the two by compressed air from lines located at the bottom of the tank. Another method would be to use paddles.

The fermentation rapidly becomes vigorous with the evolution of large quantities of carbon dioxide. In the modern plant, this gas is collected, purified, and used for the manufacture of dry ice or for other purposes. Within 50 hr., or less, the fermentation is usually complete. The fermented molasses, referred to as "beer," is distilled in a continuous still to separate the alcohol and other volatile constituents from the mash.

The alcohol is purified by means of rectifying columns and then stored in a bonded warehouse or denatured.

TABLE 25—ETHYL ALCOHOL PRODUCTION, BY KINDS OF RAW MATERIALS USED, DURING THE FISCAL YEARS 1939 TO 1947[1]

(Thousands of proof gallons. Source: Bureau of Internal Revenue)

Raw material	1939	1941	1942	1943	1944	1945	1946	1947
Grain*	15,540	17,532	38,578	107,857	206,253	281,005	104,693	39,742
Molasses*	135,884	210,427	289,396	159,190	207,523	190,201	87,118	54,159
Ethyl sulphate	47,964	69,903	90,615	96,739	113,734	111,679	127,508	133,306
Unfinished spirits and other redistillation products			5,098	81,454	45,246	32,920	23,692	49,546
Sulphate liquors						1,018	2,837	4,380
Pineapple juice	151	227	205	254*	438	241	250	220
Whey				*	136	155	136	262
Crude alcohols mixture								
Cellulose pulp and chemical mixtures	310†	576	814	1,198	1,632	1,731	1,954	2,422
Potatoes*				. . .	224		3,772	12,861
Other mixtures								
Grain, molasses, whey, pineapple juice, etc	1,218	180	97	1,095	61,366	63,791	1,296	1,983
Total alcohol produced†	201,018	298,845	424,804	447,787	636,575	683,432	353,524	298,581

[1] HIBBEN, J H, Ind Eng Chem , 40 (No 6) 990 (1948).
* Gross production, includes products used in redistillation
‡ Additional amounts used in combination with other materials included under "other mixtures"
† Cellulose pulp included in "other mixtures"

b. *The Process in Detail.* (1) TYPE OF YEAST.—Certain types of yeasts are desirable, namely, those which are able to produce and tolerate high concentrations of alcohol and which possess uniform and stable characteristics. Strains of *Saccharomyces cerevisiae* are commonly used, but other yeasts, such as *S. anamensis* and *Schizosaccharomyces pombe*, may be employed under certain conditions.

(2) PREPARATION OF STARTER —Having selected the yeast for the fermentation and having isolated it in pure culture, a starter is then prepared. A starter of large volume is required to "pitch" (inoculate) the main mash, which frequently may have a magnitude of several thousand gallons. Using aseptic technique, a tube containing about 10 cc. of sterile wort is inoculated from a pure culture of the yeast, which may be maintained on malt agar media. After incubation for a suitable period of time at a temperature of 25°C. (77°F.) to 30°C. (86°F.), the optimum for yeast growth, the culture in the tube may be used to inoculate a flask containing approximately 200 cc. of sterile mash. Following incubation, the contents of the flask may be used to seed a sterile mash of about 4-liter capacity Up to this point in the preparation of the starter, the work is

ordinarily carried out in the laboratory, using glass containers. The next mash inoculated is of semi-plant-scale size (10 to 40 gal.) and is located close to the fermenters. Usually at least one more proportionally larger mash (several hundred gallons) is inoculated and permitted to incubate. Then this fermenting mash, the starter, is either pumped or allowed to flow by gravity to the main mash. The addition of this enormous yeast culture to the mash constitutes "pitching."

Aeration is of benefit in preparing a starter, for the object is to secure an immense number of yeast cells.

The Magné automatic system or other pure culture systems (Fig. 21) for preparing the starter may be used instead of the method just outlined. In the Magné apparatus a stock of the pure culture is maintained in the upper drum of the apparatus. Mashes are inoculated from this pure culture as required, and one culture may be used over a considerable period of time before recharging with a pure culture derived from a single cell. For further details, the reader may consult the reference given below.[1]

(3) THE MOLASSES.—"Blackstrap" molasses is the principal source of industrial alcohol. This material is the sirup that is left after the recovery of crystalline sugar from the concentrated juice of sugar cane. It usually contains 18 to 55 per cent of sugars, mainly sucrose. A large part of the blackstrap molasses used in this country is imported from Cuba, although some comes from Puerto Rico.

During recent years, much "high-test" molasses has been used for ethanol manufacture. This so-called "molasses" is an evaporated sugar-cane juice that contains all the original sugar of the juice, but most of it in an inverted form as a result of acid hydrolysis. Such molasses is usually high in sugar, containing occasionally as much as 78 per cent.

(4) CONCENTRATION OF SUGAR.—A sugar concentration of 10 to 18 per cent is usually satisfactory, although other concentrations are used. When the concentration is too high it reacts adversely on the yeast, or the alcohol produced may inhibit the action of the yeast, with the consequence that the fermentation time is prolonged and some of the sugar is not properly utilized. The use of too low a concentration of sugar is uneconomic as it may lead to a loss of valuable fermenting space. Furthermore a proportionally greater quantity of fermented wort must be distilled to produce the same amount of alcohol thus adding to the expense of production.

A sugar concentration of approximately 12 per cent is frequently used. The quantity of water required to reduce the molasses to the desired sugar content is calculated from available analytical data. For

[1] Magné, J. B. P. U.S. Patent 1,212,676, Jan. 16, 1917.

FIG 24.—Pure yeast propagator. (Courtesy of The Pfaudler Co., New York City.)

example, 4 parts of water by weight would be added to 1 part of molasses, containing 60 per cent sugar, by weight, to reduce the sugar concentration to approximately 12 per cent.

The concentration of the sugar in a wort is usually determined by means of a Balling hydrometer, an instrument that gives the approximate percentage of sugar at 60°F., or 15.5°C.

(5) NUTRIENT SUBSTANCES.—Although molasses generally contains most of the nutrient substances required for fermentation, ammonium salts, such as ammonium sulphate or phosphates, may be added to the mash to supply deficiencies in nitrogen or phosphorus.

(6) pH OF THE MASH.—Fermentation proceeds satisfactorily when the mash has been adjusted to a pH of 4.0 to 4.5. This pH favors the yeast but is sufficiently low to inhibit the development of many types of bacteria. The manufacturer depends on the pH of the wort and the use of a large inoculum to take the place of sterilization, since it is costly and impracticable to sterilize large molasses mashes. Ordinarily the main mash is inoculated with a starter that represents 4 to 6 per cent of its volume, although the starter may represent from 2 to 25 per cent of the volume of the main mash under certain circumstances.

Sulphuric acid is commonly used to adjust the reaction of the mash, although lactic acid is satisfactory. Lactic acid favors the development of yeast but inhibits the growth of the butyric acid bacteria, which are detrimental to the yeast fermentation. As a substitute for the addition of acid, the mash may be inoculated with lactic acid bacteria, prior to the alcoholic fermentation.

(7) OXYGEN TENSION.—Oxygen in large amounts is necessary in the early stages for the optimum reproduction of yeast cells but is not required for the production of alcohol. During the fermentation, carbon dioxide is evolved and anaerobic conditions are soon established.

(8) TEMPERATURE.—The mash is pitched at a temperature of 60 to 80°F., usually 70 to 80°F., depending somewhat on the external temperature. During fermentation, the temperature of the mash rises. The use of cooling coils or sprays on the outside of the tank helps to maintain a suitable temperature. At temperatures much above 80°F., alcohol evaporates rather rapidly. Bacterial growth is also favored.

(9) TIME REQUIRED FOR FERMENTATION.—A fermentation is usually complete in 50 hr. or less, depending on the temperature, sugar concentration, and other factors.

(10) DISTILLATION.—The fermented mash ("beer") is distilled to separate the ethyl alcohol and fusel oil from the other constituents of the mash. In case there is a shortage of fermenters and the "beer" cannot all be distilled immediately, part of the "beer" is pumped to a storage tank, known as a "beer well," where it is held until it can be distilled

Fig. 24 — Pure yeast propagator   (Courtesy of The Pfaudler Co., New York City)

example, 4 parts of water by weight would be added to 1 part of molasses, containing 60 per cent sugar, by weight, to reduce the sugar concentration to approximately 12 per cent.

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During distillation, fractions containing different concentrations of alcohol ("wines") and slops are separated. The fractions containing approximately 60 to 90 per cent of ethyl alcohol are known as "high wines." These fractions are concentrated to 95 per cent ethanol by further distillation or fractionation. The fractions low in alcohol, the "low wines," are usually redistilled with new lots of "beer." The slops are ordinarily discarded but may be used in a number of ways. Sometimes the slops may be used as a substitute for some of the water in diluting molasses for a new mash. The solids from slops may be concentrated by heat treatment and sold as a fertilizer constituent, for they contain potassium and phosphates in addition to other components. The slops may be used as a core binder (in foundries) or as an adhesive for artificial stone (briquette)

(11) YIELD.—The common yield from blackstrap molasses amounts to approximately 90 per cent of the theoretical, on the basis of the fermentable sugars.

(12) FINAL TREATMENT.—The 190 proof ethyl alcohol (95 per cent by volume) may be further purified, dehydrated, or denatured as prescribed by the Bureau of Internal Revenue. Thus it is possible to purchase 95 per cent ethyl alcohol, with or without denaturant; e.p. (chemically pure) ethyl alcohol of 96 per cent concentration; absolute alcohol, U.S.P. (United States Pharmacopoeia); and anhydrous denatured ethyl alcohol (water-free alcohol).

(13) FLOW SHEET.—Schemes for the production of ethyl alcohol from potatoes, corn, and molasses are shown on page 128.

c. Rapid Continuous Process.—Bilford and his associates[1,2] have described a laboratory process for producing ethyl alcohol from molasses by rapid continuous fermentation. The chief value of this process lies in the fact that considerably less equipment is required than for the conventional process.

The process was briefly as follows. A suitable fermentation medium containing 10 to 15 g. of reducing sugar per 100 ml. was prepared, inoculated with an appropriate concentration of yeast cells, placed in a fermentation vessel (refer to Fig. 25), agitated with carbon dioxide (1 8 to 5.4 liters per min per liter of medium) or mechanically, and incubated at 32.2°C. During the fermentation the pH was maintained within the range of 4.5 to 5 by adjustment with ammonia at ½-hr. intervals and

[1] BILFORD, H. R, R. E. SCALF, W. H. STARK, and P. J. KOLACHOV, *Ind. Eng Chem,* **34**: 1406–1410 (1942).

[2] BILFORD, H. R, F. H. GALLAGHER, W. H STARK, and P. J. KOLACHOV, pp. 173–209, "Food for Thought" (by H. F. Willkie and P. J. Kolachov), Indiana Farm Bureau, Inc, Indianapolis, 1942.

determinations were made usually at hourly intervals of the yeast count, the Balling degree, and the reducing-sugar content of the medium. After a stationary phase of 2 5 to 5 hr., continuous operation was begun in which fresh medium was fed into the fermentor at a given rate (varied from 5 to 35 per cent per hr.) and fermented medium was withdrawn at the same rate.
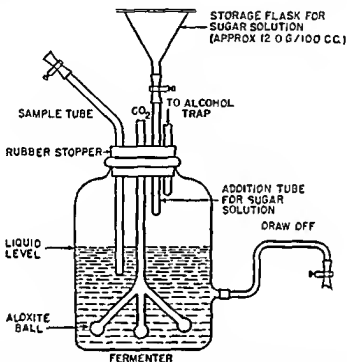
Fig 25 —Laboratory unit for continuous fermentation. [*Courtesy of H B Bilford, R E. Scalf, W H Stark, and P J Kolachov, Ind Eng Chem*, 34. 1406-1410 (1942)]

Bilford and his collaborators[1] experimented with a glucose-yeast water medium and with three molasses media. The glucose-yeast water medium was composed of 10 to 12 per cent of glucose dissolved in 10 per cent yeast water which contained 0.1 per cent of $(NH_4)_2HPO_4$. One of the molasses media contained Cuban blackstrap molasses with no supplement; the second, refined molasses plus 75 mg of $(NH_4)_2SO_4$ per 100 g of molasses; and the third, beet molasses plus 100 mg of $(NH_4)_2HPO_4$ per 100 g. of molasses. Additional data concerning the molasses media may be obtained by reference to Table 26.

The yeast used for the fermentation of the glucose-yeast water medium was a variety of *Saccharomyces cerevisiae*, designated as Seagram No. 1. An inoculum of this yeast was prepared by growing it in an

[1] Bilford, H B, R E Scalf, W H Stark, and P J Kolachov, *Ind Eng Chem*, 34: 1406 1410 (1942).

TABLE 26.—RAPID CONTINUOUS FERMENTATION OF MOLASSES[1]

Hours	pH	3 N NH4OH, ml.	Degrees Balling	Reducing sugar, g./100 ml.	Yeast, million cells/ml.	Throughput per cent total vol./hr.
A. Cuban Blackstrap, No Supplement						
0	4 92		18 0	12 02		
1	4 80	2	17 3	10 27	390	
2	4 75	2 5	15 4	8 15		Stationary phase
3	4 85	2	12 9	5 60		
4	4 80	3	9 8	2 56	550	
5	4 70	3	8 3	1 03		19
6	4 80	3	8 3	1 14	545	25
7	4 70	3	8 7	1 24	460	25
8	4 80	2	9 0	1 25	417	25
9	4 68		9 1	1 31	515	25
B Refined Molasses + 75 Mg (NH4)2SO4/100 G.						
0	5 10		18 0	13 45	315	
1	4 80	3	17 1	9 87		Stationary phase
2	4 75	5	15 4			
3	4 75	5	13 5	4 11		
4	4 56	8	11 4			
5	4 80	5	9 3	3 95	515	15
6	4 80	5	7 2	1 69		15
7	4 60	7	6 4	1 12	580	20
8	4 70	6	6 5		580	20
9	4 80	5	6 9	1 51	540	20
10	4 83	5	7 1		508	20
C Beet Molasses + 100 Mg (NH4)4HPO4/100 G						
0	4 50		18 5	10 15	350	
1	4 45		15 5	8 81	400	
2	4 50	.	12 9	6 10		Stationary phase
3	4 45	.	10 2	3 77		
4	4 35	2	7 6	1 07	530	
5	4 50	..	6 8	0 53		15
6	4 48	.	6 8			15
7	4 50	.	7 2	0 85	380	15
8	4 40	1	7 4	0 62		15
9	4 52	.	7 2	0 87	360	15
10	4 50	.	7 2	0 76	360	15
10*	4 50		7 2	0 76		15
11*	4 50		6 9	0 76	375	15
12*	4 50		7 1	0 82		15

* Mechanical agitation

[1] BILFORD, H R., R E SCALF, W H. STARK, and P. J KOLACHOV, Ind Eng Chem, 34: 1406-1410 (1942)

aerated medium for 18 hr. at 30°C. The quantity of cells required for the fermentation was separated from the growth medium by centrifuging and was resuspended in 3,000 ml of the glucose-yeast water medium. In one experiment, where the initial yeast count was 150,000,000 cells per ml., it required about 5 hr. to ferment 98 8 per cent of the sugar in a medium that before fermentation contained 10 g. of sugar per 100 ml. At this point the process was made continuous. By increasing the initial yeast count to about 350,000,000 cells per ml., it was possible to ferment 98.5 per cent of the sugar in 2 5 to 3 hr under similar conditions, after which the processes were made continuous. It was possible thus to use a throughput rate of 25 per cent per hour, during which time the reducing sugar content varied between 0 1 and 0.4 g. per 100 ml. in the fermented medium and the yeast concentration remained fairly constant at the initial cell count. Refer to Table 27.

TABLE 27 —RAPID CONTINUOUS FERMENTATION OF GLUCOSE-YEAST WATER MEDIUM[1]

Hours	pH	3 N NH₄OH, ml	Degrees Balling	Reducing sugar, g / 100 ml	Yeast, million cells/ml	Throughput per cent total vol./hr
0	5 30		10 6	10 52		Stationary phase
1	4 00	15	8 1	6 21	355	
2	4 20	10	4 0	2 39		
3	4 80	5	1 4	0 15	365	25
4	4 65	5	1 4	0 20	350	25
5	4 70	3	1 2	0 25	357	25
6	4 70	3	1 1	0 32	351	25
7	4 70	3	1 0	0 22	327	25
8	4 80	3	1 0	0 37	334	25
9	4 75	3	1 0	0 13	321	25
10	4 70	3	1 0	0 11	331	25

NOTE. Initial yeast count, approx 350 000 cells/ml , throughput rate constant

[1] BILFORD, H R , R E SCALF, W H STARK, and P J KOLACHOV, *Ind Eng Chem* , 34 1406-1410 (1942)

A different strain of yeast (*S. cerevisiae* Hansen (A T C C. 4125) was used for the fermentation of the molasses media. The inocula for these media were prepared in a manner similar to that outlined above, except that molasses media were used for the production of the cells and that it was necessary to add 5 g. of malt sprouts per 100 g. of refined molasses in addition to the ammonium sulphate Results of the experiments with the various molasses media are shown in Table 26

The yields of ethyl alcohol obtained by the continuous process were comparable to those obtained by the conventional 50-hr batch fermentation, according to Bilford and his coworkers

For further details, refer to the original paper, also to the book entitled "Food for Thought," by Willkie and Kolachov.

2. Ethyl Alcohol from Whey.—A successful method for producing ethyl alcohol from whey has been developed by Browne[1] and by Rogosa, Browne, and Whittier[2] of the Division of Dairy Research Laboratories, Bureau of Dairy Industry, U.S. Department of Agriculture.
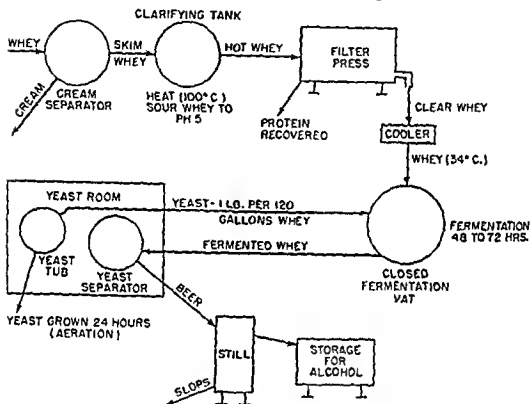
Fig. 26.—Flow sheet of alcohol production from whey. [Courtesy of M. Rogosa, H. H. Browne, and E. O. Whittier, Jour. Dairy Science, 30 (No. 4). 263 (1947).]

The process, in brief, consists of heating the whey to boiling, adjusting the pH to 5, separating out the protein by filtration, cooling the clear whey to 34°C. (93°F.), adding 1 lb of Torula cremoris per 120 gal. of whey, carrying out fermentation at 33 to 34°C. for 48 to 72 hr., separating out the yeast, and distilling the alcohol. A flow sheet of this process is shown in Fig. 26.

Since the sugar of whey is lactose, it was necessary for Rogosa and his associates[2] to examine a number of lactose-fermenting yeasts to find one that would ferment all of the lactose in a relatively short time. Their findings, in respect to whey containing 5 per cent of lactose, are illus-

[1] Browne, H. H , Ind Eng Chem . News Ed., 19 : 1271 (1941).

[2] Rogosa, M , H H. Browne, and E. O. Whittier, Jour. Dairy Sci., 30 (No. 4): 263–269 (1947)

trated in Fig. 27. It is evident from this chart that *T. cremoris* was the most satisfactory yeast examined.

The maximum amount of yeast required for seeding was found to be 2 per cent of the weight of the lactose present in the whey before fermentation.
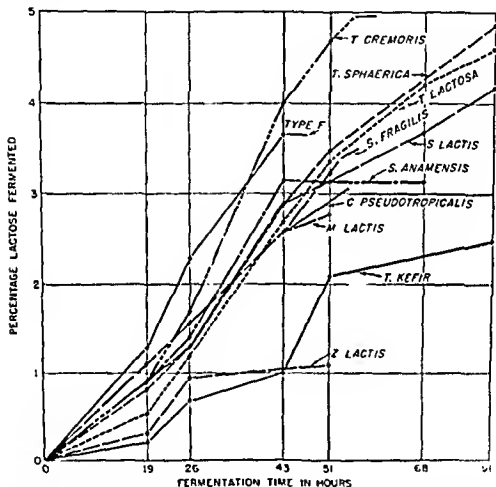
Fig 27 Relative efficiency of lactose fermentation by lactose-fermenting yeasts. [Courtesy of M Rogosa, H H Browne, and P O Walliser *Jour Dairy Science*, 30 (No 1) (1947)]

The use of a temperature of 33 to 34°C is recommended by P and his coworkers, although fermentation is actually more rapid than at any other temperature in the range of 30 to 42 C. How loss of alcohol through evaporation is more rapid also at 37°C slight savings in time accomp[...] the use of this te[...] not warranted Likewise in the [...]n of 150 gal bat[...]

started at 30°C. the temperature eventually reached a level of 33 to 34°C,
at which level it remained during the most active part of the fermentation.

Clarification may be carried out by heating the whey, adding sour
whey or acid, and filtering off the protein.   The initial pH of the clarified
whey should lie between 4.8 and 5.2.

Rogosa, Browne, and Whittier recommend that the pH of the whey

FERMENTATION TIME IN HOURS
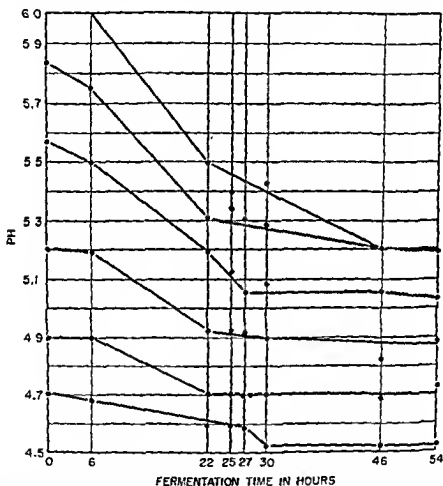
Fig. 28.—Course of pH change during fermentation by *Torula cremoris*. [*Courtesy of M. Rogosa, H. H. Browne, and E. O. Whittier, Jour. Dairy Science,* 30 (No 4). 263 (1947)]

mash be adjusted to a range of 4 7 to 5.0.   Figure 28 shows pH changes
occurring during fermentations started at different initial pH levels.

Yields of alcohol averaged 90.73 per cent on a laboratory basis, and as
low as 84 per cent under semi-plant conditions.

By-products of this fermentation are the whey protein and the slops,
the latter of which may be dried after the alcohol has been distilled in acid
solution.

The need for the following basic equipment for plant operation has

been indicated by Rogosa and his associates on the basis of their research with 150-gal batches· a cream separator, a tank equipped with heat exchanger or liquid heater for heating the whey, a chemical feeder or proportioner for handling acid or sour whey, a filter press, a cooler, closed fermentation vats (fermenters), yeast tubs and a yeast room, an air line and filter for supplying sterile air, a yeast separator, a still, a storage tank for the alcohol, facilities for concentrating and drying the slops (if desired), pumps, pipe lines, and a steam supply.

A partial list of manufacturers of some of the foregoing equipment is supplied by the U.S Department of Agriculture (BDIM-1011, May, 1947)

3. Ethyl Alcohol from Corn.—Normally corn is the chief grain source of ethyl alcohol in this country.

a. Preliminary Treatment—Corn with or without the germs is ground, mixed with water, and cooked with steam under pressure to gelatinize the starch The mash is then blown to a converter tub where additional water is added After the temperature of the mash has been reduced to about 140°F (60°C), barley malt is mixed with the mash The enzymes, contained in the malt, convert much of the starch of the corn to a fermentable sugar (maltose) and also break down some of the protein material. (Refer to the chapter on Brewing for details of the action of malt) The corn may be saccharified by the use of mold bran, acid, or by another procedure (refer to Chap III).

The mash containing the saccharified starch is transferred to a fermentor, adjusted to the proper sugar concentration by the addition of water, if necessary, cooled to a temperature of 65 to 80°F , and inoculated with yeast

b Flow Sheet—A scheme for the production of ethanol from corn is shown in Fig 29

c Some Special Uses.—Alcohol manufactured from corn is considered to be especially desirable for certain uses, on account of its freedom from foreign odors and flavors For example, such alcohol may be used in the manufacture of perfumes, flavoring extracts, and high quality medicinals.

4. Ethyl Alcohol from Wheat.—Almost 700,000,000 gal. of alcohol were produced from wheat and its products during the period from late 1942 to July, 1945, according to Boruff and Van Lanen.[1] Wheat was used as a carbohydrate source because of the huge demand for alcohol, the shortage of molasses, and the surplus of wheat during the early years of World War II   However, the distillers were not experienced in the use of wheat and often did not have the best facilities for processing it or for

[1] Boruff, C S , and J M Van Lanen, *Ind Eng Chem* , **39**: 934 (1947)
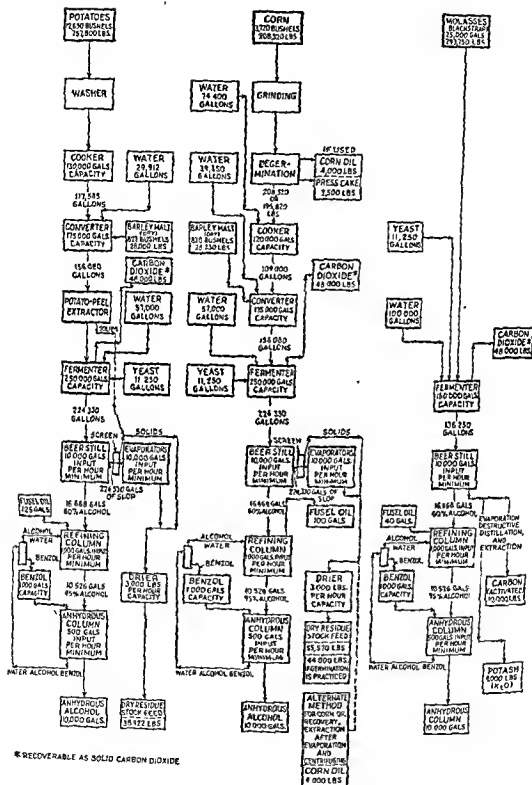
Fig. 29.—Conventional alcohol process. The residues, as shown, will be increased by the presence of yeast, yeast proteins, and unfermentable sugars. The figures actually used are based merely on the unfermentable residues calculated from the various analyses of the raw materials. (Courtesy of P. Burke Jacobs and Harry P. Newton, Industrial Farm Products Research Division, Bureau of Chemistry and Soils, U.S. Dept. Agr., Misc. Pub. 327, December, 1938.)

recovering the by-products. Furthermore, there was considerable foaming during fermentation when the concentration of wheat was high.

A Wheat-Alcohol Research Committee was appointed by the War Production Board early in World War II to help solve the problems incumbent upon the utilization of wheat. The Northern Regional Research Laboratory was selected as the coordinating agency. A summary of conferences and collaborative research was published by the laboratory in March, 1944 [1]

Stark, Kolachov, and Willkie[2] reported their findings on the use of wheat for alcohol production. They found that the White or Soft Red Winter (Red Winter subclass) types of wheat were best suited for alcohol production; that Durum and Hard Red Spring wheats were generally not suitable because of their lower starch contents; and that Hard Red Winter wheat was intermediate between these groups in acceptability. They observed that pressure-cooking by the batch or continuous-process methods or atmospheric mashing at 155°F were satisfactory.

The continuous pressure-cooking of wheat was carried out as follows: Soft Red Winter wheat (Red Winter Grade 1) was ground in such a manner that 55 per cent remained above a 20-mesh screen. The ground wheat was mixed with water and the premalt, precooked at 145°F for 10 min , passed through a jet heater at 350°F, and held for 60 sec., then cooled to 152°F. (66.7°C.), in a continuous process. In the atmospheric mashing process at 155°F , water (22 gal. per bu.) was heated to 110°F and the wheat, ground as described above, was added. The mixture was heated to 155°F in 15 min and held at this temperature for 1 hr. Thereafter the mash was cooled to the conversion temperature (145 to 148°F.) and the conversion agents were added A period of 30 min. was permitted for conversion

The problem of foaming may usually be overcome by using mixtures of corn and wheat in which the wheat represents not more than 35 to 40 per cent of the mixture.

The recovery of distillers' grain and solubles was low early in the war due to lack of adequate facilities in the distilleries, but was high by 1945. Reports concerning by-products and their recovery have been made by Miller;[3] Jacobs;[4] Boruff;[5] and others

5. Ethyl Alcohol from Dehydrated Sweet Potatoes.—The production of ethanol from dehydrated sweet potatoes of the Puerto Rico and L-1-5

[1] U S Dept of Agriculture, Northern Regional Research Laboratory, March, 1944

[2] STARK, W H , P KOLACHOV, and H F WILLKIE, *Ind Eng Chem*, 35: 133 (1943)

[3] MILLER, E S , *Am Miller*, June, 1943

[4] JACOBS, P B , Northern Regional Research Laboratory, AIC-95 (July, 1916)

[5] BORUFF, C S , *Ind Eng Chem*, 39: 602 (1947).

varieties has been studied by Jump, Zarow, and Stark.[1] The dehydrated potatoes were in the form of sticks about ⅛ to ¼ in. in diameter and up to 1½ in. in length. The potatoes, ground or unground, were prepared for fermentation by mixing with stillage and/or water, by cooking, and by saccharification with barley malt. The mash bill used in the first fermentations was usually 89 per cent dehydrated sweet potatoes and 11 per cent barley malt. Previous to cooking (at 35 gal. of water and stillage per 56-lb. bushel of potatoes), the water and stillage, in a ratio of 2.1, were heated to 120°F. The potatoes, ground or unground, were then added together with 1 per cent of the malt for premalting purposes.

Atmospheric cooking at a pH of 5.3 was accomplished during the raising of temperature of the mash to 206°F. in 1 hr., the holding of the temperature between 206 and 202°F. for 1.5 hr., and cooling to 145°F. in 5 min. Pressure-cooking, when used, was accomplished in a similar manner, except that the mash was held at 206 to 202°F. for 0.5 hr. instead of 1.5 hr. and then was autoclaved for 1 hr. at 22 lb. of steam pressure.

Saccharification was initiated at 145°F. In the first fermentations half of the malt was added and the mash was held for 10 min. at 145°F. Then the remainder of the malt was added and the mash was cooled to the setting temperature In an alternate method, all of the malt, except that used for premalting, was added and the mash was maintained at 145°F. for 60 min.

The stillage used was of two kinds: wheat-milo and sweet potato. During the first fermentations, sufficient stillage was added after the saccharification process to bring the concentration of stillage in the mash to 38 per cent.

A concentration of 45 gal. of mash per bushel of grain was used in setting the fermentors

Table 28 shows alcohol yields from ground and unground dehydrated sweet potatoes

Jump, Zarow, and Stark[2] concluded as follows as a result of their studies.

The L-4-5 variety was better than the Puerto Rico variety for alcohol production Grinding was not necessary before cooking, although it increased yields slightly. Best yields of alcohol were obtained when the final mash contained 33.3 per cent by volume of a wheat-milo stillage. Yields were distinctly lower with sweet potato stillage or with no stillage. There were no significant differences in alcohol yields from mashes cooked by atmospheric or pressure methods. In a plant run, yields of 4 77 proof gallons of alcohol per bushel were obtained with Puerto Rico sweet potatoes, and 5.44 proof gallons per bushel with the L-4-5 variety. Cooking and conversion (saccharification) of dehydrated sweet potatoes

[1] Jump, J A., A I Zarow, and W. H. Stark, *Ind. Eng. Chem.*, **36**: 1138 (1944).
[2] *Ibid.*

may be carried out successfully in grain distilleries without use of grain other than malt for conversion and without any change in the equipment

TABLE 28—ALCOHOL YIELD FROM DEHYDRATED SWEET POTATOES, GROUND AND UNGROUND[1,2]

Potato	Grind	Balling	Final data			
			Sugar, g / 100 ml	Alcohol yield, proof gal /bu		Plant efficiency, per cent
				Wet	Dry	
L-4-5 (shredded)	Unground	0 6	0 72	6 06	6 50	92 5
	Coarse	0 6	0 80	6 16	6 61	94 1
	Fine	0 6	0 80	6 16	6 61	94 1
Puerto Rico (shredded)	Unground	1 7	0 78	5 76	6 27	86 6
	Coarse	1 9	0 96	5 70	6 32	87 3
	Fine	1 8	0 85	5 76	6 27	86 7
Puerto Rico	Unground	1 2	0 76	5 76	6 33	87 3
	Coarse	1 2	0 76	5 88	6 47	89 2
	Fine	1 2	0 75	5 89	6 48	89 3

[1] JUMP, J A, I ZAROW and W H STARK, Ind Eng Chem , 36, 1138 (1944)
[2] Represents the average of three fermenters

6. Ethyl Alcohol from Jerusalem Artichokes.—The Jerusalem artichoke, or girasol (*Helianthus tuberosus*), native to North America, is a plant that has been studied in recent years as a possible source of levulose and/or industrial alcohol. Although this plant is cultivated on a large scale in some parts of Europe, it is not an important crop in the United States at present, except in a few localities.

The Jerusalem artichoke is rich in the polysaccharide inulin ($C_6H_{10}O_5$)$_n$, which is readily hydrolyzed to levulose

a Yield per Acre.—The Jerusalem artichoke has been studied by Boswell and his associates[1] in considerable detail Investigating 20 varieties of the Jerusalem artichoke, all grown in three different parts of the United States for three different years, they found that the mean yield per acre was 6.58 tons at Urbana, Ill., 16.73 tons at Corvallis, Ore , and 8 74 tons at Washington, D C. The mean yield of the 20 varieties at all three places for three years was 10.69 tons per acre

b. Sugar Content—The sugar content of the tubers, after hydrolysis, varied in different seasons. A six-year mean analysis of the 20

[1] BOSWELL, V R , C E STRINHAUER, M F BABB, W L BURLISON, W H ALDERMAN, and H A SCIOTH, *U S Dept Agr , Tech Bull.* 514, May, 1936

varieties of Jerusalem artichokes investigated above showed 13 33 per cent levulose and 16.38 per cent total sugars.

c. Storage.—If an alcohol plant is to be operated continuously a constant supply of the raw material must be available. The fresh Jerusalem artichoke tuber cannot be stored satisfactorily. However, certain methods for storing the product have been worked out. Mc-Glumphy and his associates[1] showed that the thinly sliced tuber could be satisfactorily desiccated and stored without sugar loss.

Both the dried and fresh tuber chips may be extracted by water in diffusion batteries, but such extracts deteriorate rapidly owing to micro-organisms. If the extract, which contains the soluble sugars, is evaporated under reduced pressure to a total solid content of greater than 70 per cent, it becomes immune to decomposition by bacteria and yeasts By storing the concentrated sirup under an atmosphere of carbon dioxide, mold growth is also prevented.[2]

The carbohydrates of the extract were stable at pH values between 4.8 and 9.0 at temperatures as high as 110°C.[2]

d. Fermentation.—For fermentation, the sirup is diluted to yield approximately 12 per cent reducing sugars after hydrolysis, sterilized, cooled, and inoculated with yeast. *Saccharomyces cerevisiae, S. anamensis,* and especially *S. pombe* have been used with satisfactory results by Underkofler and his associates.[2]

It is not necessary to make a preliminary hydrolysis of the sirup or to add additional nutrient substances

e. Culture of Yeast.—When the yeast was continuously cultured on unhydrolyzed sirup from artichokes, its ability to produce ethanol was increased.[2]

f. Yields—Yields of 90 per cent or more were obtained under the foregoing conditions.

7. **Ethyl Alcohol from Sulphite Liquor.**—Sulphite liquor is produced as a waste product in the manufacture of pulp from wood.

a. Pulping Process.—Spruce, hemlock, or some other kind of wood is cut into small chips that are subsequently digested with calcium bisulphite, using heat and pressure. The sulphite liquor reaches a temperature of 130 to 140°C. toward the end of the process. In some processes the temperature may rise even higher, with the result that some of the sugar in the liquor is destroyed. The cellulose pulp obtained by this method is used for the manufacture of paper.

[1] McGlumphy, J. H., J. W. Eichinger, R. M. Hixon, and J. H. Buchanan, *Ind. Eng Chem*, **23:** 1202 (1931)

[2] Underkofler, L A, W. K. McPherson, and E. I Fulmer, *Ind. Eng Chem*, **29:** 1160 (1937).

b. Composition of Sulphite Liquor.—Together with each ton of pulp, there is produced 8 to 10 tons of sulphite liquor, which contains from 10 to 12 per cent of total solids One analysis[1] of sulphite liquor showed the presence of the following products for each 1,000 kg. of cellulose obtained from Swedish spruce: lignin, 644 kg.; carbohydrates, 311 kg.; proteins, 15 kg.; resin and fats, 73 kg.; sulphur dioxide combined with lignin, 235 kg.; and calcium oxide combined with lignosulphonic acid, 102 kg

The carbohydrates consisted of 49.4 per cent glucose, 15 6 per cent mannose, 8.1 per cent galactose, and 26 9 per cent nonfermentable pentosans (arabinose).

Approximately 65 per cent of the total reducing sugars are usually fermentable.

c. Treatment of Sulphite Liquor—Before the liquor is inoculated with yeast it is necessary to remove or neutralize the sulphur dioxide, acetic acid, and formic acid that are present. The methods commonly employed to accomplish this are (1) steam-stripping followed by neutralization with lime or calcium carbonate; or (2) direct neutralization.

d. Production.—The production of ethyl alcohol from sulphite waste liquor has been described by Foth, Sankey, and Rosten (1944), Joseph (1947), Ericsson (1947), and others.

Ethyl alcohol is produced commercially from sulphite liquor in this country, Canada, Germany, Sweden, and other countries.

e. Process of Bellingham, Wash—Ericsson[2] described the production of ethyl alcohol from sulphite waste liquor at the Bellingham plant. Figure 30 shows a flow diagram for the process.

COLLECTION OF LIQUOR.—Sulphite waste liquor from the digesters is discharged, together with wood pulp fibers, into blow pits that have perforated bottoms or plates of stainless steel The sulphite liquor is drained off and stored in a tank at a temperature of about 90°C

CONDITIONING.—From the storage tank the sulphite liquor is pumped to the top of a column and flows downward over stainless-steel perforated plates, while steam introduced at the bottom of the column and flowing upwards removes sulphur dioxide and other volatile materials. The sulphur dioxide saved is reused. The hot sulphite liquor is pumped through screens which remove wood pulp fibers. It flows by gravity to a storage tank. Passage of the liquor through flash coolers reduces its temperature to 30°C and concentrates it by about 10 to 12 per cent A slurry of lime is added to adjust the pH to 4.5. Urea is added to the liquor as a source of nitrogen.

[1] EWLSON, E. W., *Chem Inds*, **58**: 573 (1936).
[2] ERICSSON, E. O., *Chemical Eng Progress, Trans Sect*, **43** (No. 4): 165 (1947)
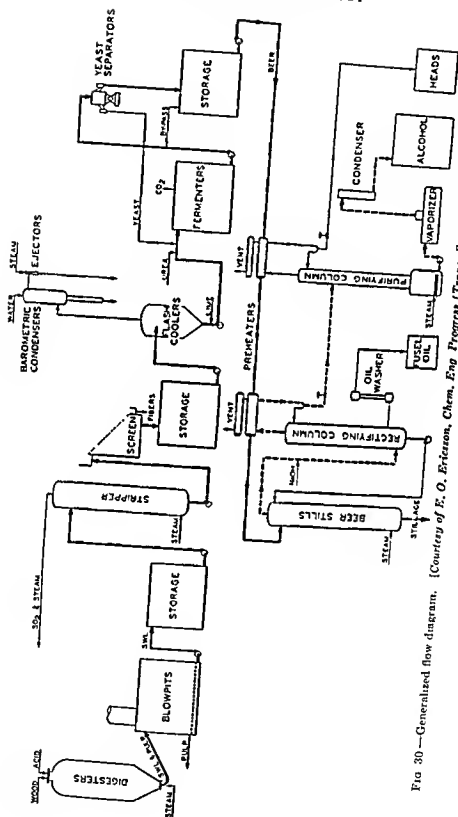
Fig. 30 —Generalized flow diagram. [Courtesy of E. O. Ericsson, Chem. Eng. Progress (Trans. Sect.), 43 (No. 1): 165 (1917).]

FERMENTATION.—The conditioned sulphite liquor is pumped into the first of a series of seven interconnected fermentation tanks. Yeast from a previous cycle is mixed with the sulphite liquor as it flows to the fermenter. The medium is agitated in each tank and the flow of liquor is on a continuous basis. Fermentation is carried out at 30°C.

SEPARATION OF YEAST.—After the fermentation is over, in about 20 hr., the fermented liquor is pumped to yeast separators. The required amount of yeast is returned to the first fermenter for reuse.

FINAL OPERATIONS.—The clarified liquor (beer) is passed by gravity to a storage tank. Then it is distilled. Finally the alcohol is rectified to 190 proof or higher, purified, and warehoused.

YIELD.—The yield is 22 gal. of alcohol per ton of pulp.

CAPACITY OF PLANT.—The capacity of the plant is 6,600 gal. (or more) of alcohol per day.

f. The Melle Process.—This process was developed by Les Usines de Melle[1] and involves the "reuse of yeast." The yeast from a completed alcohol fermentation is separated from the alcohol and spent liquor by means of centrifugal separators. This yeast is used again and again with usually no appreciable loss of fermentation ability. The reuse of yeast is particularly important in the alcoholic fermentation of sulphite liquor, where large quantities of yeast are essential for rapid and efficient fermentations.

8. Ethyl Alcohol from Wood Sugars.—In order to obtain maximum yields from wood sugars, it is essential to remove inhibiting substances and to add necessary nutrients.

Several actions may be taken to remove inhibiting materials. For example, Leonard and Hajny[2] found that the toxic character of the wood-sugar solutions could be counteracted by the addition of reducing agents, such as Na_2SO_3, $NaHSO_3$, $Na_2S_2O_3 \cdot 5H_2O$, $Na_2S_2O_5$, $Na_2S_4O_6$, $KHSO_3$, Na_2S, sulphite waste liquor, alkali decomposed sugar, ascorbic acid, cysteine, and reduced-iron filings. The same effect could be secured by heating neutral solutions. The amount of dissolved calcium sulphite was decreased by neutralization at about 140°C. For good alcohol yields and avoidance of sugar losses, heating should be for 15 to 30 min. at 140°C. with the solution at a pH of 4.5 to 5.2. Toxic substances were removed from the wood hydrolyzate by steam distillation, which was particularly beneficial when a quantity of greater than 0.1 per cent of furfural was present.

Leonard and Hajny[2] found the following method of treating wood

[1] Canadian Patents 341,720, 348,549; 402,547.
[2] LEONARD, R. H., and G. J. HAJNY,   R 1468, U.S. Dept. Agr., Forest Products Lab., December, 1944.

fermenting wood sugars to be successful: The acid hydrolyzate was neutralized at room temperature to a pH of 5.0 ± 0.2 with a slurry of calcium carbonate. The product was filtered and 0.03 per cent by weight of sulphur dioxide as Na_2SO_3, $Na_2S_2O_3$, or similar reducing agent, was added. The mixture was heated for 15 min. at 135 to 140°C. in a bomb, drawn, and cooled to 30°C. The pH of the medium was adjusted to 5.8 with sodium hydroxide. Nutrients, 0.02 per cent urea and 0.006 per cent NaH_2PO_4 by weight, were added and the medium was inoculated with 2 per cent by volume of fresh yeast (S. cerevisiae No. 49 of the University of Wisconsin collection) and agitated. The fermentation was usually complete in less than 20 hr. when the initial sugar concentration, as glucose, was 5 to 7 g. per 100 ml.

Harris and associates[1] studied the fermentation of Douglas fir hydrolyzates. They observed that the fermentation time was 5 to 6 hr. when the inoculum was 2 per cent by dry weight of S. cerevisiae (No. 49 University of Wisconsin) and 21 hr. when the inoculum was 0.5 per cent by dry weight. The yeast from an 18- to 20-hr. fermentation was used to inoculate a fresh lot of medium containing 5 per cent sugar. At the end of this fermentation the yeast was separated out and used to inoculate a new batch. This sequence was followed 59 times without loss in alcohol yield and without contamination with bacteria and other yeasts or molds. It was possible to increase the rate of fermentation but not the yield of alcohol from wood sugars by the addition of 0.025 per cent Louisiana second-crop molasses. They obtained yields of 39.2 to 40 per cent based on total sugar and 47.0 to 47.9 per cent based on fermented sugar. In terms of a 5 per cent sugar solution, a yield of 2.64 gal. of 95 per cent alcohol per 100 gal. of hydrolyzate was obtained. From 1 ton of dry bark-free wood, 64.5 gal. of alcohol were produced.

Two-bushel-per-Day Continuous Alcohol Unit.—Altsheler and his associates[2] have designed a continuous-process unit capable of producing 5 gal. of 190-proof alcohol in 24 hr. from 2 bu. of grain. A flow diagram of the process employed is shown in Fig. 31. The process is as follows:

Corn is transported from a bin by a screw-conveyor to a Raymond hammer mill where it is ground in such a manner that substantially all of it passes through a 20-mesh screen. The meal is blown by the mill to a cyclone separator from which it falls to a slurry vessel made of Pyrex glass. It is mixed with enough water to provide a ratio of about 23 gal.

[1] HARRIS, E. E., G. J. HAJNY, M. HANNAN, and S. ROGERS, Mimeo No. R 1618, U.S. Dept. of Agriculture, Forest Products Lab., June, 1946.

[2] ALTSHELER, W. B., H. W. MOLLET, E. H. C. BROWN, W. H. STARK, and L. A. SMITH, Chem. Eng. Progress, Trans. Sect., 43 (No. 9): 467 (1947).

per bu. Sufficient sulphuric acid is added to produce a weight concentration of 1.25 per cent.

The mash is forced to a cooker tube, which is 1 5 in. in diameter and made of copper, by a piston pump. Steam at a pressure of 150 lb. per sq in. heats it almost instantly to 340°F. It is held in the cooker for 6 min , and then neutralized by a slurry of calcium carbonate. It is then cooled to 88 to 90°F. in a heat exchanger (20 ft. of 0.25-in copper tubing in the form of a helix with cooling water on the outside), and discharged to the first of two fermenters
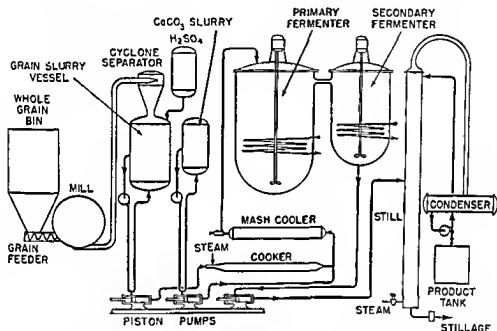
Fig 31.—Simplified process flow diagram [*Courtesy of W B Alsheler, H W Mollet, E H C Brown, W H. Stark, and L A Smith, Chem Eng Progress (Trans Sect), 43 (No. 8): 467 (1947).*]

Fermentation is continuous and is carried out in Pyrex-glass vessels each of which is equipped with a stirrer The primary fermenter is equipped with cooling coils and has a working capacity of 27 gal It is connected at the top with the secondary fermentor by means of an overflow pipe. The mash remains about 8 hr. in the primary fermentor and about 3 hr in the secondary fermentor.

The concentration of the mash as it enters the primary fermentation tank is 1 bu. of corn in 40 gal. Yeast is added to the mash when the process is first started, but the rate of reproduction is sufficiently rapid thereafter to obviate the necessity of further additions. The usual concentration of cells is 100,000,000 per milliliter The temperature is

TABLE 29.—AVERAGE ANNUAL PRODUCTION OF CERTAIN AGRICULTURAL BY-PRODUCTS FOR 1931–1935[1]

By-product	Grain per bushel, pounds	Dry by-product per pound of grain, pounds	Dry by-product per bushel of grain, tons	Average production of dry by-product, 1,000 tons	Estimated quantity of dry by-product available for industrial use, 1,000 tons
Wheat straw	60	1.9	0 057	38,794	29,000
Rye straw	56	2.5	0 070	2,378	1,800
Oat straw	32	1 3	0.0208	20,156	0
Barley straw	48	1.2	0 0283	5,965	4,474
Flax straw	56	4.0	0 112	1,128	1,128
Rice straw	45	1 2	0.027	1,089	820
Total straws.			. . .	69,510	37,222
Corncobs	56	0 22	0.00610	12,408	1,400
Oat hulls	32	0 30	0 0048	4,651	150
Rice hulls	45	0.20	0 0045	182	182
Cottonseed hulls	..		.....	1,165*	583
			...	183†	92
Total cobs and hulls	.	...	. ...	18,589	2,407
Corn stover	56	1 2	0.0336	63,681	28,500
Cotton stems and pods	..	....	‡	17,544‡	12,281
Bagasse fiber, continental United States				423§	423
Bagasse fiber, insular United States				3,276‖	3,276
Total other by-products.....	..	....	....	84,924	44,480
Grand total	..	. .	.... .	173,023	84,109

[1] Senate Document 65, 76th Congress, 1st Session, "A Report of a Survey Made by the Department of Agriculture Relative to Four Regional Research Laboratories, One in Each Major Farm Producing Area," Washington, D.C., 1939.

maintained at 88 to 90°F. in the primary fermentor by means of water circulating through coils.   The pH is 4.4 to 4.6.

Alcohol is stripped from the beer by 20 perforated plates located in the lower section of the still and rectified by 26 bubble-cap plates in the upper section of the still.   The alcohol vapors from the top of the column travel to a condenser, which is cooled by water.

For further details, the reader is referred to the original publication by Altsheler and his coworkers.[1]

Cost of Producing Ethyl Alcohol.—The cost of producing ethyl alcohol from various raw materials varies considerably and is determined more or less by world conditions. Tousley[2] has discussed this subject in some detail.

Cellulosic Wastes.—Each year millions of tons of cellulose-containing materials are permitted to go to waste in the United States. In Table 29 are presented estimates concerning the production of various by-products, which may be termed agricultural wastes or residues. These data are, of course, subject to variation from year to year, since crop production is not stable.

TABLE 30—AVERAGE YIELD[1] OF 99 5 PER CENT ALCOHOL PER TON[1]

Material	Gallons	Material	Gallons
Wheat (all varieties)	85 0	Yams	27 3
Corn	84 0	Potatoes	22 0
Buckwheat	81 4	Sugar beets	22 1
Raisins	81 4	Figs, fresh	21 0
Grain sorghum	70 5	Jerusalem artichokes	20 0
Rice, rough	70 5	Pineapples	15 6
Barley	79 2	Sugar cane	15 2
Dates, dry	79 0	Grapes (all varieties)	15.1
Rye	78 8	Apples	14.4
Prunes, dry	72 0	Apricots	13 6
Molasses, blackstrap	70 4	Pears	11.5
Sorghum cane	70 4	Peaches	11.5
Oats	63 6	Plums (nonprunes)	10.9
Figs, dry	59 0	Carrots	9 8
Sweet potatoes	34 2		

[1] Probable yield from a short ton of the raw material, calculated from the average fermentable content

[2] JACOBS, P. B., and H. P. NEWTON, *U S Dept Agr*, *Misc Pub* 327, December, 1938

Yields from Various Raw Materials.—The probable average yield of 99 5 per cent ethyl alcohol per ton of raw material is shown in Table 30.

Table 31 supplies information concerning the probable average yield of ethyl alcohol per acre from various farm crops

Government Supervision.—The purpose of Government supervision is to prevent the illegal use of untaxed alcohol. Ethyl alcohol is taxed, of course, to provide revenue for the Government. Should tax-free ethyl alcohol be used for beverage purposes, the tax from such alcohol would be lost.

[1] *Ibid*

[2] TOUSLEY, R D , *Chem Met Eng*, **52** (No 10)· 120 (October, 1945)

composition and the quantity of fusel oil formed varies, however, ac
ing to the raw materials used and the nature of the fermentation.
example, 1 gal. of fusel oil may be obtained for each 1,000 gal. of et
in the fermentation of molasses,[1] but larger quantities may be obt
from potatoes and corn.

Fusel oil is used principally as a lacquer solvent. It is usuall
refined or separated into its components.

A further discussion of some of the constituents of fusel oil w
found in the following chapter.

References on the Production of Industrial Alcohol and Related Subject:

ALTSHELEN, W. B., H W MOLLET, E. H C BROWN, W. H. STARK, and L. A. S
Design of a Two-bushel per Day Continuous Alcohol Unit, *Chem. Eng. Pr*
Trans Sect., 43 (No 9): 467–472 (1947).

ANON.: Alcohol on the Hoof, Seagram's Designs Mobile Continuous Process Al
Unit, *Chem. Inds*, October, 1946.

ANON. Former Waste Product Now Yields 7,500 Gallons of Alcohol Daily, *De*
Centrifugal Rev, 13 (No. 2). 3–4 (1947).

ANON: General Plans of Operation and List of Equipment for Whey Fermentatic
Yeast, BDIM-1011, *Bureau of Dairy Industry, U.S. Dept. Agr.*, May,

ATWATER, W O., and A. P. BRYANT· The Chemical Composition of American
Materials, *U.S Dept Agr , Bull.* 28, rev. ed., 1906

BARRON, H : "Distillation of Alcohol," *Division of Education*, Joseph E. Seagr
Sons, Inc., Louisville, Ky., 1944.

BENNHAUER, K : "Gärungschemisches Praktikum," 2d ed., Verlag Julius Spri
Berlin, 1939.

BILFORD, H. B., R E. SCALF, W. H. STARK, and P J KOLACHOV: Alcoholic Ferme
tion of Molasses, Rapid Continuous Fermentation Process, *Ind. Eng. Cl*
34: 1406–1410 (1942).

BORUFF, C. S , and J M VAN LANEN· The Fermentation Industry during World
II, *Ind Eng. Chem*, 39: 934–937 (1947).

BOSWELL, V R , C E STEINBAUER, M F BABB, W. L BURLISON, W. H. ALDER
and H. A SCHOTH· Studies on the Culture and Certain Varieties of the Jerus
Artichoke, *U.S Dept Agr , Tech. Bull* 514, May, 1936.

BROWNE, H. H : Ethyl Alcohol from Fermentation of Lactose in Whey, *Ind. I*
Chem., News Ed., 19: 1271 (1941)

CHATFIELD, C , and G. ADAMS: Proximate Composition of Fresh Vegetables, l
Dept. Agr , Circ 146, 1931.

—— and L I. McLAUGHLIN: Proximate Composition of Fresh Fruits. *U.S. D*
Agr., Circ. 50, 1928.

CLAASSEN, H.: Die Wirtschaftlichkeit der Herstellung von Holzzucker, *Chem. l*
55: 989 (1932).

COLEMAN, D. A (chairman): "Methods for the Analysis of Cereals and Cereal Pr
ucts Reference Tables," *American Association of Cereal Chemists*, Lancas
Pa , 1928.

DE BECZE, G., and M. ROSENBLATT: Continuous Fermentation, *Am. Brewer*,
(No. 2): 11 (1943)

[1] JACOBS, P B., and H. P NEWTON, *U S Dept Agr , Misc Pub.* 327, Decemb
1938.

DUNN, P. M.: Industrial Alcohol from Wood Waste, *Chem. Products*, November–December, 1945.

ERICSSON, E. O.: Alcohol from Sulfite Waste Liquor, *Chem. Eng. Progress, Trans. Sect.*, 43 (No. 4): 165–167 (1917).

EWESON, E. W.: Yeast from Wood, *Chem. Inds*, 38: 673 (1936).

FAITH, W. L, and J. A. HALL: Ethyl Alcohol from Waste Wood by a Modified Scholler Process, *Chem. & Eng. News*, 22 (No 7): 525–526 (1944)

FOTH, G.: "Handbuch der Spiritus fabrikation," Chap. 19, Paul Parey, Berlin.

GIORDANI, M.: Alcool da materiali cellulosici, *Chimica e industria (Italy)*, 21: 265–272 (1939).

GRAY, W. D.: The Acclimatization of Yeast to High Concentrations of Glucose: The Subsequent Effect on Alcohol Tolerance, *Jour Bact*, 52 (No 6): 703 (1946).

HARRIS, E E.: Industrial Alcohol from Wood Waste, *Southern Lumberman*, Dec 15, 1945.

———, G. J. HAJNY, M. HANNAN, and S. C. ROGERS: Fermentation of Douglas Fir Hydrolyzate by *S. cerevisiae*, *Ind. Eng. Chem*, 38: 890–901 (1946), also *Mimeo. No. R1618*, U.S. Dept Agr., Forest Products Laboratory, Madison, Wis, June, 1946.

JACOBS, P. B.: Alcohol from Agricultural Commodities, *Report No AIC-95*, U.S. Dept. Agr., revised, February, 1917

——— and H P NEWTON: Motor Fuels from Farm Products, *U S. Dept Agr., Misc Pub 327*, December, 1938.

———, R. K. WRIGHT, and F. M HILDEBRANDT: General Purpose Fermentation Plant, *Ind. Eng. Chem.*, 40 (No 4): 750–764 (1948)

JOSEPH, H. G.: Industrial Alcohol Production from Sulfite Waste Liquor by the Ontario Paper Co, Ltd, *Sewage Works Jour*, 19 (No 4): 60–69 (1947).

KRESSMAN, F W : The Manufacture of Ethyl Alcohol from Wood Waste, *U S Dept Agr*, *Bull* 983, 1922.

LEONARD, R. H, and G. J HAJNY Fermentation of Wood Sugars to Ethyl Alcohol, *Ind. Eng. Chem*, 37 : 390–395 (1945)

LES USINES DE MELLE: *Canadian Patents* 344,720 318,519, and 402, 347,

LICHTY, L. C, and C. W PHELPS Gasoline-alcohol Blends in Internal Combustion Engines, *Ind. Eng. Chem*, 20: 222 (1938)

LIEBMANN, A. J.: Alcohol in War, *Wallerstein Labs Communs*, 5 (No. 15): 333–357 (1942).

McGLUMPHY, J. H., J W EICHINGER, R M HIXON, and J H BUCHANAN: Commercial Production of Levulose, *Ind Eng Chem*, 23: 1202 (1931).

MEHTA, T. N.: "Power Alcohol for India," Dept Chemical Technology, University of Bombay, Bombay, India, 1945.

MILLER, D. S.: Industrial Alcohol from Wheat, *Am Miller*, June, 1943

OWEN, W. L.: Continuous Fermentation, *Sugar*, 43 (No 2): 36 (1948)

ROGOSA, M., H. H BROWNE, and E O WHITTIER: Ethyl Alcohol from Whey, *Dairy Science*, 30 (No. 4): 263–269 (1947)

RUF, E W, W H STARK, L A SMITH, and E E ALLEN: Alcohol from Wood Waste, *Ind Eng Chem*, 40 (No ...

SAEMAN ...... Nov. 14, 1945

SANKEY, C A, and M M ROSTEN: Alcohol .... Waste Liquor .... No of Pulp & Paper Mag Can (1944

Hexose Phosphates.—Harden and Young[1] (1905) discovered the importance of phosphates in cell-free fermentation. They have demonstrated that inorganic phosphates disappear during the first part of the fermentation, while organic phosphates, *i.e.*, esters of hexose, are produced. They have described hexosediphosphate and proposed the following fundamental equation for fermentation by yeast extract:

$$2C_6H_{12}O_6 + 2PO_4HR_2 = 2C_2H_5OH + 2CO_2 + C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O$$
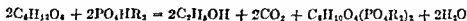
Hexosediphosphate is an important intermediate in the schemes of Embden, Meyerhof, and others for the breakdown of carbohydrates. This compound contains two phosphate, or phosphoric acid, molecules attached, one each, to the first and sixth carbon atoms of the hexose which appears to be fructose. The same hexosediphosphate is obtained from glucose, fructose, or mannose.

At least two hexosemonophosphates, which have different properties, have been prepared: the Robison ester (glucopyranose-6-phosphoric ester), and the Neuberg ester (fructofuranose-6-phosphoric ester). When these esters are hydrolyzed, they yield glucose and fructose, respectively.[2]

Hexosemonophosphates have been synthesized by Levene and Raymond, Smythe, and others. A trehalosemonophosphate has been isolated by Robison and Morgan from a fermentation brought about by dried yeast.

Hexose phosphates are fermented at different rates. The diphosphate is fermented more slowly than glucose, while hexosemonophosphate is usually fermented more rapidly than the diphosphate, at a rate comparable with glucose in the initial stage only.[3]

The addition of hexose phosphates to cell-free yeast extracts containing glucose removes or greatly shortens the period of induction (the pause before fermentation starts). Without phosphates, no fermentation takes place.

The addition of phosphates to a medium does not affect the rate of fermentation by living yeasts.

For further details in respect to this very important subject, the reader is referred to Harden's monograph, "Alcoholic Fermentation," and to the bibliography that follows this chapter.

Methods of Studying the Mechanism of Fermentation.—Information concerning the mechanism of the ethyl alcohol fermentation may be

[1] *Jour. Physiol.*, **32** (Proc., Nov. 12, 1904), 1905

[2] GORTNER, R. A., "Outlines of Biochemistry," 2d ed., John Wiley & Sons, Inc., New York, 1938

[3] MICHAELIS, L., *Ind. Eng. Chem.*, **27**: 1037 (1935)

gained by studying the related mechansim of lactic acid formation by muscle extracts; by the use of cell-free yeast juice or extracts, fixation methods, selective poisons, and dialysis; and by other means.

1. *Related Mechanism of Lactic Acid Formation by Muscle Extracts.*— Any productive advance in the study of the mechanism of lactic acid formation by muscle extract has usually aided materially in the study of the mechanism of the ethyl alcohol fermentation, and vice versa. The researches of Embden on muscle extract exemplify the impetus that research on the former may do for the latter. The outstanding work of Meyerhof and his school add further proof to the valuable insight gained through correlative studies.

2. *Cell-free Extracts.*—Since the important reactions that take place in the conversion of sugar to ethyl alcohol occur normally within the living cell, since added hexose phosphates are fermented extremely slowly or not at all, and since the isolation of intermediates from the living cell is impossible in many cases, but comparatively little information concerning the intermediary reactions may be derived from studying such cells Accordingly, cell-free extracts are used

Two common methods of preparing the extract are the Buchner method (already mentioned) and the Lebedev[1] method In the latter method the yeast is washed thoroughly with water, dried at 25 to 30°C., rubbed through a sieve, dried further, and stored until such time as an extract may be required. The extract is prepared by mixing 1 part by weight of dried yeast with 3 parts of water, incubating at 37°C for 3 hr. and filtering the extract into a vessel cooled by ice.[2] It is important to use an extract that is free from living cells.

3. *Fixation Method.*—The fixation method was used with success by Neuberg. In this method a sulphite, such as calcium sulphite, or dimedon (dimethyl cyclohexane-dione), is used to fix the acetaldehyde as it is formed. The fixation product is not fermented by yeast and therefore accumulates in the medium. Since acetaldehyde is a hydrogen acceptor, the removal of this substance from the fermentation medium causes other hydrogen acceptors to become active Use is made of this fact in the production of glycerol by fermentation, where the role of sulphites is very important.

Although pyruvic aldehyde forms an addition product with sulphites, this product is fermentable.

4. *Selective Poisons.*—Certain reactions in the normal chain of enzyme reactions do not take place in the presence of selective poisons, such as

[1] Lebedev, A. von , *Compt rend*, **152**: 49, 1129 (1911)

[2] Stephenson, M., "Bacterial Metabolism," 2d ed , Longmans, Green & Company, New York, 1930

TABLE 33.—SOME IMPORTANT INTERMEDIATE AND END PRODUCTS OF THE ETHYL ALCOHOL FERMENTATION

I. *Intermediate Products*

$CH_2O(PO_3H_2)$
$HOCH$
$HOCH$
$HCOH$
HC
$CH_2O(PO_3H_2)$
Fructofuranose
1,6-diphosphate

CH_2OH
HOC
$HOCH$
$HCOH$
HC
$CH_2O(PO_3H_2)$
Neuberg ester

$HOCH$
$HCOH$
$HOCH$
$HCOH$
HC
$CH_2O(PO_3H_2)$
Robison ester

CH_2OH
$C=O$
$CH_2O(PO_3H_2)$
Dihydroxyacetone phosphate

CHO
$CHOH$
$CH_2O(PO_3H_2)$
3-Glyceraldehyde phosphate

CH_2OH
$CHOH$
$CH_2O(PO_3H_2)$
α-Glycerol phosphate

$COOH$
$CHOH$
$CH_2O(PO_3H_2)$
3-Phospho-glyceric acid

$COOH$
$CHO(PO_3H_2)$
CH_2OH
2-Phospho-glyceric acid

$COOH$
$CO(PO_3H_2)$
CH_3
Phospho-pyruvic acid

$COOH$
$C=O$ or
CH_3
Pyruvic acid

$COOH$
COH
CH_2
Pyruvic acid (enol form)

CH_3
$C=O$
CHO
Methylglyoxal

CHO
CH_3
Acetaldehyde

II. *End Products*

A. Derived from Sugar

CH_3
CH_2OH
Ethyl alcohol

CO_2
Carbon dioxide

CH_2OH
$CHOH$
CH_2OH
Glycerol

$COOH$
CH_3
Acetic acid

B. Not Derived from Sugar

CH_3
$CHCH_2OH$
CH_3CH_2
d-Amyl alcohol

CH_3
$CHCH_2$ CH_2OH
CH_3
Isoamyl alcohol

CH_2COOH
CH_2COOH
Succinic acid

In case the reaction is alkaline, a compound, believed to be unstable and of the formula $C_3H_6O_3$, acts as a hydrogen acceptor together with acetaldehyde. The unstable $C_3H_6O_3$ compound is reduced to glycerol, while acetaldehyde is oxidized in part to acetic acid and reduced in part to ethanol. (A Cannizzaro reaction.)

SCHEME II

$$2C_6H_{12}O_6$$

$2C_3H_6O_3$ $\qquad$ $2C_2H_4O_2$
$-2H_2O$ $\qquad$ $+2H_2$

$2CH_3 CO\ \overset{O}{\underset{H}{C}}$ $\qquad$ $2C_3H_8O_3$ (Glycerol)

$+O_2$

$2CH_3 CO\ COOH$

$2CH_3\ \overset{O}{\underset{H}{C}}$ $+\ 2CO_2$

$+\frac{1}{2}O_2$ $\qquad$ $+H_2$
CH_3COOH $\qquad$ CH_3CH_2OH
Acetic acid $\qquad$ Ethanol
(Neuberg's "third form" of fermentation)

One method of producing glycerol is by the sulphite fixation process. Acetaldehyde may be fixed by sulphite before it has become reduced or oxidized The aldehyde thus fixed may be recovered from the fermentation medium and estimated. It is evident that any acetaldehyde that has become fixed is no longer available as a hydrogen acceptor. Acetaldehyde may, then, be replaced in part by $C_3H_6O_3$ as a hydrogen acceptor. $C_3H_6O_3$ is reduced to glycerol in proportion to the amount of acetaldehyde fixed (1 molecule of glycerol for each molecule of acetaldehyde fixed).

SCHEME III

$$C_6H_{12}O_6 \rightarrow \begin{cases} C_3H_6O_3 & +H_2 \rightarrow C_3H_8O_2 \\ C_3H_6O_3 & -H_2O \rightarrow CH_3CO\ CHO + \frac{1}{2}O_2 \rightarrow CH_3COCOOH \end{cases}$$

$CH_3\ \overset{O}{\underset{H}{C}}\ + CO_2$

(fixed)
(Neuberg's "second form" of fermentation)

The normal fermentation, according to Neuberg, proceeds chiefly as shown in the first scheme, but some glycerol and acetic acid occur and hence the second scheme is realized in part.

KLUYVER'S SCHEME FOR THE ALCOHOLIC FERMENTATION OF GLUCOSE[1]

I. *Initial Phosphorylation*

$$
\begin{array}{ccc}
\begin{array}{l}
\text{—CHOH} \\
\quad | \\
\text{CHOH} \\
\quad | \\
\text{O} \quad \text{CHOH} \\
\quad | \\
\text{CHOH} \\
\quad | \\
\text{—CH} \\
\quad | \\
\text{CH}_2\text{OH} \\
\text{d-Glucose}
\end{array}
& + HR_2PO_4 \rightarrow &
\begin{array}{l}
\text{CHO} \\
\quad | \\
\text{CHOH} \\
\quad | \\
\text{CHOH} \\
\quad | \\
\text{CHOH} \\
\quad | \\
\text{CHOH} \\
\quad | \\
\text{CH}_2\text{O·PO}_3\text{R}_2 \\
\text{Active hexosemono-} \\
\text{phosphate}
\end{array}
& + H_2O \longrightarrow &
\begin{array}{l}
\text{—CHOH} \\
\quad | \\
\text{CHOH} \\
\quad | \\
\text{O} \quad \text{CHOH} \\
\quad | \\
\text{CHOH} \\
\quad | \\
\text{—CH} \\
\quad | \\
\text{CH}_2\text{O·PO}_3\text{R}_2 \\
\text{Robison's} \\
\text{ester}
\end{array}
\end{array}
$$
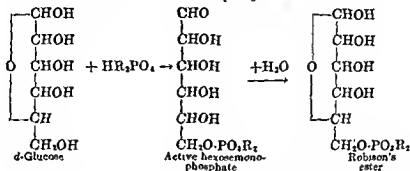
II. *Oxidoreduction of Hexosemonophosphate*

$$
\begin{array}{l}
\text{CHO} \\
\quad | \\
\text{CHOH} \\
\quad | \\
\text{CHOH} \\
\quad | \\
\text{CHOH} \\
\quad | \\
\text{CHOH} \\
\quad | \\
\text{CH}_2\text{O PO}_3\text{R}_2 \\
\text{Active hexosemono-} \\
\text{phosphate}
\end{array}
\rightarrow
\begin{array}{l}
\text{CHO} \\
\quad | \\
\text{CHOH} \\
\quad | \\
\text{CH}_2\text{OH} \\
\text{Glyceraldehyde} \\
+ \\
\text{CHO} \\
\quad | \\
\text{CHOH} \\
\quad | \\
\text{CH}_2\text{O PO}_3\text{R}_2 \\
\text{Glyceraldehyde} \\
\text{monophosphate}
\end{array}
$$
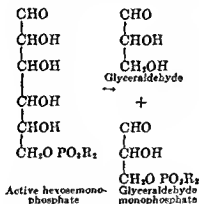
III. *Hydrolysis of Triosephosphate*
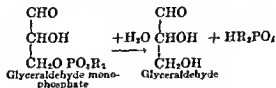
$$
\begin{array}{l}
\text{CHO} \\
\quad | \\
\text{CHOH} \\
\quad | \\
\text{CH}_2\text{O PO}_3\text{R}_2 \\
\text{Glyceraldehyde mono-} \\
\text{phosphate}
\end{array}
+ H_2O
\begin{array}{l}
\text{CHO} \\
\quad | \\
\text{CHOH} \\
\quad | \\
\text{CH}_2\text{OH} \\
\text{Glyceraldehyde}
\end{array}
+ HR_2PO_4
$$

Condensation Reaction

$$
\begin{array}{l}
\text{CHO} \\
\quad | \\
\text{CHOH} \\
\quad | \\
\text{CH}_2\text{O PO}_3\text{R}_2
\end{array}
+
\begin{array}{l}
\text{CHO} \\
\quad | \\
\text{CHOH} \\
\quad | \\
\text{CH}_2\text{O·PO}_3\text{R}_2
\end{array}
\rightarrow
\begin{array}{l}
\text{CH}_2\text{O·PO}_3\text{R}_2 \\
\quad | \\
\text{CHOH} \\
\quad | \\
\text{CHOH} \\
\quad | \\
\text{C=O} \\
\quad | \\
\text{CHOH} \\
\quad | \\
\text{CH}_2\text{O PO}_3\text{R}_2 \\
\text{Hexosediphosphate of} \\
\text{Harden and Young}
\end{array}
$$
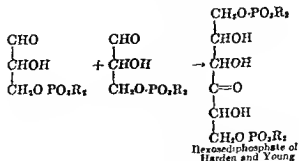
[1] KLUYVER, A. J., Die bakteriellen Zuckervergärungen, Ergeb Enzymforsch, 4: 230–273 (1935).

KLUYVER'S SCHEME FOR THE ALCOHOLIC FERMENTATION OF GLUCOSE.[1]—(*Continued*)
IV. *Final Oxidoreductions*

A.

$$\begin{array}{ccccc}
\text{CH}_2\text{OH} & & \text{CH}_2\text{OH} & & \text{CH}_2\text{OH} & \text{CH}_3 & \text{CH}_3 \\
\text{CHOH} +\text{H}_2\text{O} & \text{CHOH} & -\text{H}_2\text{O} & \text{C-H} & \text{C=O} & \text{C=O} \\
\text{CHO} & \rightarrow & \text{OH} & \rightarrow & \text{OH} & \rightarrow \text{OH} & \rightarrow \text{OH} \\
& & \text{C-OH} & & \text{C-OH} & \text{C-OH} & \text{C-OH} \\
& & \text{H} & & \text{H} & \text{H} & \text{H}
\end{array}$$

Glyceraldehyde   Hydrated glyceraldehyde   Hydrated methylglyoxal

B.

$$\begin{array}{ccccc}
\text{CH}_3 & & \text{CH}_3 & & \text{CH}_3 & \text{CH}_3 \\
\text{C=O} + & \text{CH}_3 & \text{(Acceptor,} & \text{C=O} + & \text{CH}_3 \\
\text{OH} & \text{CHO} & \text{in general)} & \text{COOH} & \text{CH}_2\text{OH} \\
\text{C-OH} \\
\text{H}
\end{array}$$

Hydrated methylglyoxal   Acetaldehyde   Pyruvic acid   Ethanol

C.

$$\begin{array}{ccc}
\text{CH}_3 & \text{CH}_3 \\
\text{C=O} & \rightarrow & \text{CH}_3 + \text{CO}_2 \\
\text{COOH} & \text{CHO}
\end{array}$$

Pyruvic acid   Acetaldehyde   Carbon dioxide
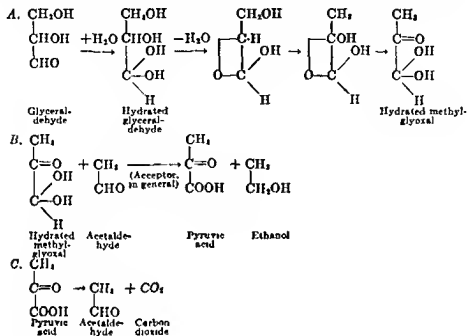
[1] KLUYVER, A J., *Die bakteriellen Zuckervergärungen*, *Ergeb. Enzymforsch.*, **4**: 230–273 (1935).

Methylglyoxal has been isolated from the fermentation of hexose-diphosphate by yeast extracts in water and toluol. It has likewise been isolated as a "semicarbazido" in the fermentation of sucrose by yeasts. It is not fermentable but may be regarded as the stable isomer of a fermentable form.[1]

An active hexosemonophosphate is first formed by the combination of glucose with a phosphate. Two 3-carbon compounds, glyceraldehyde and glyceraldehyde monophosphate, are the main end products formed through the splitting of the active hexosemonophosphate, but Robison's ester may be produced from this compound as a side product.

Glyceraldehyde monophosphate is hydrolyzed principally to glyceraldehyde and phosphate, while 2 molecules may condense by a side reaction to form the hexosediphosphate of Harden and Young. The phosphate liberated in the hydrolysis of glyceraldehyde monophosphate is available to react with more glucose to form more active hexosemonophosphate.

Glyceraldehyde is converted to **hydrated methylglyoxal** through a series of hydrogen transfer reactions. Methylglyoxal hydrate donates

hydrogen to a hydrogen acceptor and is changed to pyruvic acid. Acetaldehyde acts as the hydrogen acceptor after it is once formed, being reduced to ethanol, while pyruvic acid is being produced. Pyruvic acid is decarboxylated to acetaldehyde and carbon dioxide. The acetaldehyde reacts with more methylglyoxal hydrate and the production of pyruvic acid, ethanol, acetaldehyde, and carbon dioxide continues.

Kluyver[1] believes that the catalytic agents responsible for the

Fig. 32 —Scheme for fermentation

important changes in this scheme for the fermentation of glucose are an oxidoreductase and a phosphatase.

Scheme Based on the Researches of Meyerhof and Others.—In Fig. 32 a scheme for the intermediate reactions occurring in the ethyl alcohol fermentation is shown.

The first step, in the series of reactions, is a phosphorylation of the sugar (glucose, fructose, or mannose) in which a phosphorylating coenzyme, known as the adenylic system, and obtainable in a yeast dialyzate, function.[2] This system, or coenzyme, consists of adenylic acid (adenosinemonophosphate), adenosinediphosphate and adenosinetriphosphate. The first two of these compounds have the ability to pick up phosphate, while the third, adenosinetriphosphate, donates phosphate. The formulas for two important members of the adenylic system follow:

---

[1] For a further discussion of this subject, the reader is referred to A. J. Kluyver. "The Chemical Activity of Microorganisms," University of London Press, Ltd., 1931.

[2] MEYERHOF, O., *Nature*, 141: 855 (1938)

Adenylic acid, or Adenosinemonophosphate

Adenosinetriphosphate

The hexose accepts phosphate from adenosinetriphosphate, the hexosemonophosphate appearing first,[1] then the hexosediphosphate upon further phosphorylation. Once the fermentation has reached the pyruvic acid stage, the phosphate given off by phosphopyruvic acid becomes available for phosphorylating additional hexose.

Fructose diphosphate, or diphosphoric acid (the common diphosphate obtained from glucose, fructose, or mannose), splits to form 2 molecules of triosephosphate with which it is in equilibrium:
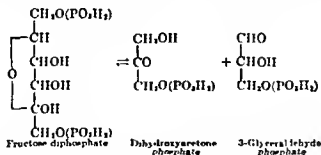
3-Glyceraldehyde is in equilibrium with dihydroxyacetone and is converted largely to the latter, according to Meyerhof[1]
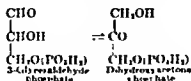
[1] *Ibid*

During the initial stages of fermentation (the induction period), before any acetaldehyde is produced, 2 molecules of triosephosphate dismutate to form 1 molecule of 3-phosphoglyceric acid and 1 molecule of α-glycerophosphate, this being an oxidation-reduction reaction in which cozymase (coenzyme I) is active:

$$
\begin{array}{ccccc}
CH_2OH & CHO & & CH_2OH & COOH \\
| & | & & | & | \\
CO & + CHOH & \xrightarrow[\text{mase}]{+H_2O \atop (\text{cozy-})} & CHOH & + CHOH \\
| & | & & | & | \\
CH_2O(PO_3H_2) & CH_2O(PO_3H_2) & & CH_2O(PO_3H_2) & CH_2O(PO_3H_2) \\
\text{Dihydroxy-} & \text{3-Glyceralde-} & & \alpha\text{-Glycer-} & \text{3-Phospho-} \\
\text{acetone} & \text{hyde phosphate} & & \text{phosphate} & \text{glyceric} \\
\text{phosphate} & & & & \text{acid}
\end{array}
$$
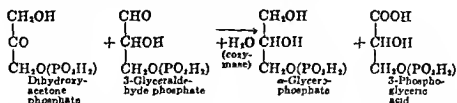
α-Glycerophosphate is hydrolyzed to glycerol and phosphoric acid:

$$
\begin{array}{ccc}
CH_2OH & CH_2OH & \\
| & | & \\
CHOH & + H_2O \quad CHOH & + H_3PO_4 \\
| & | & \\
CH_2O(PO_3H_2) & CH_2OH & \\
\alpha\text{-Glycero-} & \text{Glycerol} & \text{Phosphoric} \\
\text{phosphate} & & \text{acid}
\end{array}
$$
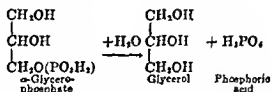
The preceding reaction accounts for the small amount of glycerol that is produced in the normal ethyl alcohol fermentation.

3-Phosphoglyceric acid proceeds, through a series of reversible enzyme reactions, to break down through 2-phosphoglyceric acid to phosphopyruvic acid:

$$
\begin{array}{ccc}
COOH & COOH & COOH \\
| & | & | \\
CHOH & \rightleftharpoons CHO(PO_3H_2) \rightleftharpoons CO(PO_3H_2) & + H_2O \\
| & | & | \\
CH_2O(PO_3H_2) & CH_2OH & CH_2 \\
\text{3-Phosphoglyceric} & \text{2-Phosphoglyceric} & \text{Phosphopyru-} \\
\text{acid} & \text{acid} & \text{vic acid}
\end{array}
$$
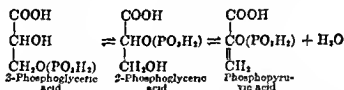
The addition of sodium fluoride to a yeast extract containing either 3-phosphoglyceric acid or 2-phosphoglyceric acid prevents the formation of phosphopyruvic acid, for the enzyme (enolase) is poisoned.

Phosphopyruvic acid is dephosphorylated by adenylic acid to form pyruvic acid, the adenylic acid taking up phosphate to become adenosinetriphosphate.

$$
\begin{array}{ccc}
COOH & & COOH \\
| & +2H_2O & | \\
2CO(PO_3H_2) + \text{Adenylic acid} & \xrightarrow{\quad\quad} & 2COH \quad + \text{Adenosinetriphosphate} \\
| & & | \\
CH_2 & & CH_2 \\
\text{Phosphopyruvic} & & \text{Pyruvic acid} \\
\text{acid} & & \text{(enol form)}
\end{array}
$$

$$
\begin{array}{c}
COOH \\
| \\
2CO \\
| \\
CH_3 \\
\text{Pyruvic acid} \\
\text{(Adenylic system reaction)}
\end{array}
$$
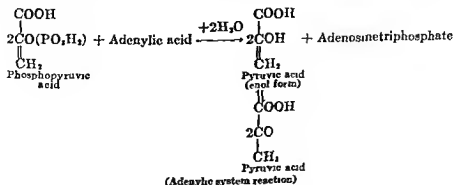
Adenosinetriphosphate donates phosphate to 2 molecules of hexose to form 2 molecules of hexosemonophosphate and 1 molecule of adenylic acid, the net result being expressed by the following reaction:

Phosphopyruvic acid + Hexose → Pyruvic acid + Hexosemonophosphate
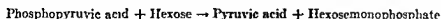
Additional phosphopyruvic acid is broken down in the same manner with the result that the hexosemonophosphate is further phosphorylated:

Phosphopyruvic acid + Hexosemonophosphate → Pyruvic acid + Hexosediphosphate

In this manner, hexosediphosphate arises during the stationary phase of fermentation.

Pyruvic acid is broken down to acetaldehyde and carbon dioxide by the enzyme carboxylase:

$$
\begin{array}{l}
\text{COOH} \\
| \\
\text{CO} \\
| \\
\text{CH}_3 \\
\text{Pyruvic acid}
\end{array}
\xrightarrow{\text{Carboxylase}}
\begin{array}{l}
\text{CHO} + \text{CO}_2 \\
| \\
\text{CH}_3 \\
\text{Acetalde-\ Carbon} \\
\text{hyde\ dioxide}
\end{array}
$$
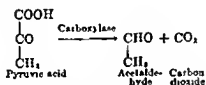
When present in sufficient quantity, acetaldehyde reacts with reduced cozymase (coenzyme I) with the result that acetaldehyde is reduced to ethyl alcohol while cozymase is oxidized. The oxidized cozymase now reacts with the triosephosphate (glyceraldehyde phosphate), oxidizing it to 3-phosphoglyceric acid, while cozymase becomes reduced again. It is thus seen that cozymase acts in the capacity of a hydrogen carrier in this oxidation-reduction. This reaction occurs normally during the stationary phase of fermentation

$$
\begin{array}{l}
\text{CHO} \\
| \\
\text{CHOH} \\
| \\
\text{CH}_2\text{O(PO}_3\text{H}_2) \\
\text{3-Glyceraldehyde} \\
\text{phosphate}
\end{array}
+
\begin{array}{l}
\text{CHO} \\
| \\
\text{CH}_3 \\
\text{Acetalde-} \\
\text{hyde}
\end{array}
\rightarrow
\begin{array}{l}
\text{COOH} \\
| \\
\text{CHOH} \\
| \\
\text{CH}_2\text{O(PO}_3\text{H}_2) \\
\text{3-Phospho-} \\
\text{glyceric acid}
\end{array}
+
\begin{array}{l}
\text{CH}_2\text{OH} \\
| \\
\text{CH}_3 \\
\text{Ethyl} \\
\text{alcohol}
\end{array}
$$

(Cozymase oxidation-reduction reaction)
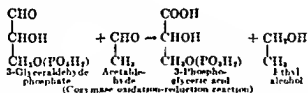
The phosphoglyceric acid thus formed is broken down through the series of reactions outlined above to produce more acetaldehyde, which reacts with more triosephosphate to produce more phosphoglyceric acid. The cycle continues in this manner to the end of the fermentation.

Types of Reactions.—Meyerhof[1] recognizes four main types of reactions in the foregoing scheme for the intermediate reactions of the ethyl alcohol fermentation. These include the phosphorylation-dephosphorylation reactions, the oxidation-reduction reactions, the reversible reactions, and the decarboxylation reaction.

The adenylic system, the mechanism of which has already been

[1] *Ibid*

explained, is concerned in the phosphorylation-dephosphorylation reactions.

Oxidoreductases and cozymase (coenzyme I) are responsible for the conversion of the triose phosphates to α-glycerophosphoric acid and 3-phosphoglyceric acid during the induction period and for the oxidation of triosephosphate (glyceraldehyde phosphate) with the simultaneous reduction of acetaldehyde during the stationary period of fermentation. Nicotinic acid amide[1] is the hydrogen-carrying group of cozymase. The function of cozymase in transporting hydrogen may be illustrated by the following equation in which R represents the ribose phosphoric acid group of cozymase (see structural formula on p. 41) and R_1 represents the adenosinemonophosphate group:

$$ \text{Cozymase} + H_2 \rightleftharpoons \text{Reduced cozymase (Dihydrocozymase)} $$
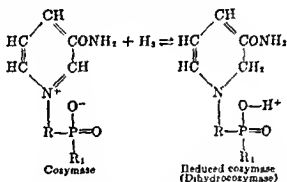
The principal reversible reactions include the reaction between hexosediphosphate and 2 molecules of triosephosphate; the reaction between dihydroxyacetone phosphate and glyceraldehyde phosphate; and the reactions between 3-phosphoglyceric acid, 2-phosphoglyceric acid, and phosphopyruvic acid.

A summary of the reversible reactions appears in the following scheme of Meyerhof.[1] The relation of each intermediate or end product to its immediate predecessor is indicated.

Glucose (Fructose, etc)
$\downarrow$ + 1H$_3$PO$_4$
Glucose-6-phosphoric acid $\rightleftharpoons$ Fructose-6-phosphoric acid
$\downarrow$ + 1H$_3$PO$_4$
Fructose-(-1-6-) phosphoric acid
$\uparrow\downarrow$

Dihydroxyacetone phosphate   3-Glyceraldehyde phosphate
$-H_2 \downarrow \uparrow + H_2$ $\downarrow + O$
α-Glycerolphosphoric acid   3-Phosphoglyceric acid
$\downarrow$ $\uparrow\downarrow$
Glycerol + H$_3$PO$_4$ 2-Phosphoglyceric acid
          $\uparrow\downarrow$
          Phosphopyruvic acid + H$_2$O
          $\downarrow$
          Pyruvic acid + H$_3$PO$_4$
          $\downarrow$
          Acetaldehyde + CO$_2$
          $\downarrow$
          Ethyl alcohol
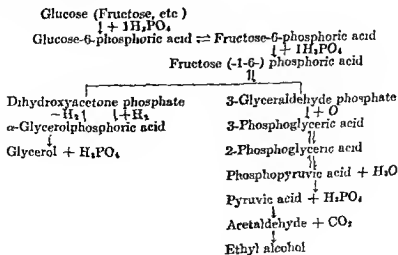
[1] *Ibid*

Some Enzymes Active during Fermentation.—Several enzymes catalyze the intermediate reactions of the ethyl alcohol fermentation. Hexokinase converts fermentable hexoses to more active forms. Zymohexase catalyzes the breakdown of hexosediphosphate to triosephosphates. Oxidoreductase (mutase, dehydrase) with cozymase plays a very important role in the oxidation-reduction reactions. The conversion of phosphoglyceric acid to pyruvic and phosphoric acids is catalyzed by enolase, while carboxylase breaks down pyruvic acid to form acetaldehyde and carbon dioxide

In Table 34 (Cori, 1942) are shown reactions catalyzed by 12 separate enzymes and dialyzable components required for the action of some of these enzymes.

TABLE 34.—ALCOHOLIC FERMENTATION[4]

(Intact yeast cell: glucose = 2CO$_2$ + 2 alcohol, cell-free juice: 2 glucose + 2KH$_2$PO$_4$
= 2CO$_2$ + 2 alcohol + 1 hexosediphosphate)

Non-dialyzable enzymes and reactions catalyzed	Dialyzable components
1 Glucose + ATP → glucose-6-P + ADP[5]	*Mg + + ions needed for reaction*
2 Glucose-6-P ⇌ fructose-6-P	ATP = Adenosinetriphosphate
3 Fructose-6P + ATP → fructose-1-6-diP + ADP[5]	ADP = Adenosinediphosphate
4 Fructose-diP ⇌ dihydroxyacetone-P + glyceraldehyde-P	
5 Dihydroxyacetone-P ⇌ glyceraldehyde-P	
6 3-glyceraldehyde-P + KH$_2$PO$_4$ + Pyr ⇌ 1-3-glyceric acid-P + H$_2$Pyr ¹	Pyr. = Pyridine-nucleotide H$_2$Pyr. = Reduced pyridine-nucleotide
7 1-3-glyceric acid-P + ADP ⇌ 3-glyceric acid-P + ATP[5]	KH$_2$PO$_4$ = Inorganic phosphate
8 3-glyceric acid-P ⇌ 2-glyceric acid-P[5]	
9 2-glyceric acid-P ⇌ pyruvic acid-P + H$_2$O[5]	
10 Pyruvic acid-P + ADP → pyruvic acid + ATP[5]	
11 Pyruvic acid + DPT → acetaldehyde + CO$_2$	DPT = Diphosphothiamin
12 Acetaldehyde + H$_2$Pyr ⇌ alcohol + Pyr[3]	

Note. Owing to the splitting of the sugar molecules in two halves in reaction 4, reactions 6 to 12 should be multiplied by two to balance the equation given above for intact yeast cells.

¹ Cori C F *Proc 5th Ann Meet Am Soc Brewing Chemists* May 25 27, 1942.

[5] Enzymes obtained in crystalline form.

Basis for Theory.—Space permits only a relatively brief discussion of the facts upon which the foregoing theory is based. Many of the important discoveries were made during investigations of the intermediate reactions concerned in the formation of lactic acid in muscle

extract and were subsequently established for the ethyl alcohol fermentation.

Parnas, Lutwak-Mann, and Mann have demonstrated the importance of adenylic acid and adenosinetriphosphate as dephosphorylating and phosphorylating agents, respectively.

Hexosediphosphate has been isolated repeatedly and is readily fermented by yeast and muscle extracts.

Hexosediphosphate enters into equilibrium with the 2 molecules of triosephosphate (Meyerhof and Lohmann, 1934): dihydroxyacetone phosphate, synthesized by Kiessling, and 3-glyceraldehyde phosphate, synthesized by H. O. L. Fischer (1932). These two triosephosphates have been isolated from muscle extract and subsequently from yeast extract fermentations of glucose or of hexosediphosphate with the aid of monoiodoacetic acid. They are readily fermented by yeast extract and together form an equilibrium in which dihydroxyacetone phosphate predominates. Whether one starts with a mixture of synthetic or natural triosephosphates or with hexosediphosphate a reversible reaction quickly occurs between hexosediphosphate and the triosephosphates in the presence of yeast extract.

Important information concerning the 3-carbon intermediates has been obtained by the use of sodium fluoride and monoiodoacetic acid. Embden (1933), using a muscle extract poisoned with sodium fluoride, showed that phosphoglyceric acid was an intermediate compound. He showed that in the absence of fluoride, phosphoglyceric acid was converted to pyruvic and phosphoric acid through enzymic action. Nilsson isolated phosphoglyceric acid from a fermentation by yeast extract.

Meyerhof and Kiessling showed that there were two phosphoglyceric acids: 3-phosphoglyceric acid and 2-phosphoglyceric acid. These acids have been isolated from muscle extract and synthesized by Kiessling. They differ in structure, solubilities, and optical rotations.

Phosphopyruvic acid was isolated by K. Lohmann as a crystalline salt. It has been synthesized by Kiessling.

Pyruvic acid has been isolated as an intermediate and is readily fermented by living yeasts or yeast extracts. It is converted to acetaldehyde and carbon dioxide by the enzyme carboxylase, discovered by Neuberg.

Acetaldehyde has been demonstrated by fixation with sulphites and dimedon.

The Origin of Amyl and Isoamyl Alcohols.—Ehrlich[1] has shown that amyl and isoamyl alcohols are derived from the amino acids, namely,

[1] HARDEN, A , "Alcoholic Fermentation," 4th ed , Longmans, Green & Company, New York, 1932.

isoleucine and leucine, respectively.   These acids are obtained from the medium usually, but in cases of nitrogen deficiency may originate from yeast protein.

$$CH_3$$
$$CH \cdot CH(NH_2) \, COOH + H_2O \rightarrow$$
$$CH_3 \cdot CH_2$$
Isoleucine

$$CH_3$$
$$CH \cdot CH_2OH + CO_2 + NH_3$$
$$CH_3 \cdot CH_2$$
d-Amyl alcohol

$$CH_3$$
$$CH \cdot CH_2 CH(NH_2) \, COOH + H_2O \rightarrow$$
$$CH_3$$
Leucine

$$CH_3$$
$$CH \cdot CH_2 CH_2OH + CO_2 + NH_3$$
$$CH_3$$
Isoamyl alcohol
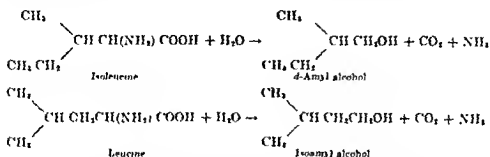
The formation of these alcohols from their corresponding amino acids may be demonstrated by inoculating sterile mashes, containing a fermentable sugar and a measured quantity of one or both of the acids, with a pure culture of yeast and analyzing the mashes after the fermentation for the amino acids and alcohols.   Control experiments should be carried out at the same time, of course, with mashes that do not contain added amino acids.

The ammonia set free in the foregoing reactions is utilized immediately by the yeast cells.

The relative proportions and likewise the quantities of the two alcohols formed depend on the composition of the medium, *i.e.*, on the relative amounts of the two amino acids, the presence or absence of ammonium salts, and the nature of the sugar; on the species of yeast; on the nutritive condition of the yeast; and on other factors.   Certain ammonium salts cause a diminution in the normal yield of amyl alcohols, ammonia being derived from the salts rather than from the amino acids

Leucine and isoleucine are not converted to their corresponding alcohols by living yeast cells in the absence of a fermentable sugar, nor are they converted by yeast juice or by zymin (a product prepared by treating yeast with alcohol and ether, or by acetone and ether)

Alcohols are produced from other alpha-amino acids in a similar manner by yeasts   Such products are believed to contribute to the flavor of beer, wine, rum, and other alcoholic beverages   For example, tyrosol, the alcohol produced from tyrosine, possesses a bitter taste and is concerned in the flavor of beer

Succinic Acid ($HOOC \cdot CH_2 \cdot CH_2 \cdot COOH$).—Succinic acid is believed to arise from glutamic acid during fermentation   Ehrlich discovered (1909) after experimentation with several amino acids, which included aspartic acid ($HOOC \cdot CH_2 \cdot CH(NH_2) \, COOH$), that glutamic acid was

the only amino acid added to the fermentation medium that produced a well-defined increase in the quantity of succinic acid.

The probable course of its formation from glutamic acid is as follows:[1]

$$HOOC \cdot CH_2 \cdot CH_2 \, CH(NH_2) \, COOH \quad \text{(Glutamic acid)}$$
$$\downarrow \tfrac{1}{2}O_2 \quad \text{(Oxidative deamination)}$$
$$NH_3 + HOOC \cdot CH_2 \cdot CH_2 \cdot CO \; COOH \quad \text{(α-Ketoglutaric acid)}$$
$$\downarrow \quad \text{(Decarboxylation)}$$
$$CO_2 + HOOC \cdot CH_2 \; CH_2 \; CHO \quad \text{(Succinic semialdehyde)}$$
$$\downarrow \tfrac{1}{2}O_2 \quad \text{(Oxidation)}$$
$$HOOC \; CH_2 \cdot CH_2 \; COOH \quad \text{(Succinic acid)}$$
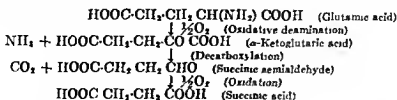
In the absence of added nitrogen-containing substances, succinic acid may be produced from the glutamic acid derived from the autolysis of the protein of yeast.

Succinic acid is not formed by yeast in the absence of sugar nor is it produced by yeast juice or zymin.

References on the Mechanism of the Ethyl Alcohol Fermentation

ANDERSON, C. G.: "An Introduction to Bacteriological Chemistry," William Wood & Company, The Williams & Wilkins Company, Baltimore, 1938

ANHAGEN, E.: Co-Carboxylase, ein neues Co-Enzym der alkoholischen Gärung, *Zeit. physiol Chem.*, 204: 149 (1932).

CORI, C. F.: The Enzymatic Reactions in Alcoholic Fermentation, *Proc 5th Ann. Meeting Am. Soc Brewing Chem.*, May 25–27, 1942.

EMBDEN, G., H J. DEUTICKE, und G. GRAFT: Über die intermediären Vorgänge bei der Glykolyse in der Muskulatur, *Klin Wochschr.*, 12: 213 (1932).

EULER, H VON: Neuere Ergebnisse an enzymatischen Oxydations- und Reduktions-Systemen, *Ergeb Enzymforsch*, 3: 135 (1934).

——— und K. MYRBÄCK· Co-Zymase und Adenylsäure, *Zeit. physiol. Chem.*, 199: 189 (1931).

——— und R. NILSSON· Adenosintriphosphorsäure und Co-Zymase, *Zeit. physiol. Chem*, 208: 173 (1932)

FISCHER, H. O L., und E BAER· Über die 3-Glycerinaldehyde-phosphorsäure, *Ber*, 65: 337 (1932)

FREY, C. N.: History and Development of Modern Yeast Industry, *Ind. Eng Chem*, 22: 1154 (1930).

GODA, T : Beitrag zur enzymatischen Oxydoreduktion zwischen Glycerinphosphor-saure und Brenztraubensaure, *Biochem Zeit*, 297: 347 (1938)

———. Kann lebende Hefe Hexosen direkt vergaren? *Biochem. Zeit.*, 298: 431 (1938)

HARDEN, A : "Alcoholic Fermentation," 4th ed , Longmans, Green & Company, New York, 1932.

——— and R ROBISON: A New Phosphoric Ester Obtained by the Aid of Yeast-juice (Preliminary note), *Proc Chem Soc.*, 30: 16 (1914)

——— and W J. YOUNG· The Influence of Phosphates on the Fermentation of Glycose by Yeast Juice (Preliminary communication), *Proc Chem Soc*, 21: 189 (1905)

[1] *Ibid.*

HOPKINS, R. H., and C. KRAUSE: "Biochemistry Applied to Malting and Brewing," D Van Nostrand Company, Inc, New York, 1937.

KIESSLING, W.: Über die Synthese der Dioxy-aceton-phosphorsäure *Ber.*, 67: 869 (1934).

———: Über die Reindarstellung von Glucose-1-phosphorsäure (Cori-Ester), *Biochem. Zeit.*, 298: 421 (1938).

——— und O. MEYERHOF Über ein Adenindinucleotid der Hefe: Di-(Adenosin-5'-phosphorsäure), *Biochem Zeit*, 298: 410 (1938).

KLUYVER, A. J: "The Chemical Activities of Micro-organisms," University of London Press, London, 1931.

———: Die bakterellen Zuckervergärungen, *Ergeb Enzymforsch*, 4: 230 (1935)

LEBEDEV, A.: Sur l'extraction de la zymase, *Compt rend*, 152: 1129 (1911)

LEHMANN, J. Aktivierung von Hefealkoholdehydrogenase durch Co-Enzym, *Biochem Zeit*, 272: 95 (1934)

———: Zur Kenntnis biologischer Oxydations-Reduktions-potentiale Vorläufige Potentialmessungen im System. Alkohol-Dehydrogenase-Acetaldehyd, *Biochem. Zeit.*, 274: 321 (1934)

———: Über den Mechanismus der Vergärung des Dioxyacetons, *Biochem Zeit*, 277: 261–267 (1935)

LEVENE, P A., and A L RAYMOND: Hexosemonophosphates Glucose-3-Phosphate, Glucose-6-Phosphate and Their Bearing on the Structure of Robison's Ester, *Jour Biol Chem*, 89: 479 (1930)

——— and ———: Hexosemonophosphate (Robison) Natural and Synthetic, *Jour. Biol Chem*, 91: 751 (1931)

——— and ———: Hexosemonophosphate Synthetic Robison Ester, *Jour Biol Chem*, 92: 757 (1931)

——— and ———: Hexosemonophosphates Galactose-6-Phosphate, *Jour Biol Chem*, 92: 765 (1931)

LOHMANN, K: Über die Isolierung verschiedener natürlicher Phosphorsäureverbindungen und die Frage ihrer Einheitlichkeit, *Biochem Zeit*, 294: 306 (1928)

——— und P SCHUSTER: Untersuchungen über die Co-carboxylase, *Biochem Zeit*, 294: 188 (1937)

——— und ———: Über die Co-carboxylase, *Naturwissenschaften*, 25: 26 (1937)

LUTWAK-MANN, C, und T MANN: Über die Verkettung der chemischen Umsetzungen in der alkoholschen Gärung, I, *Biochem Zeit*, 281: 140 (1935)

MEYERHOF, O Intermediate Products and the Last Stages of Carbohydrate Breakdown in the Metabolism of the Muscle and in Alcohol Fermentation, *Nature*, 132: 337, 373 (1933)

———: Sur les processus intermédiaires dans la dégradation des glucides (formation d'acid lactique et fermentation alcoolique), *Ann Inst Pasteur*, 53: 221 (1934)

——— Über die Intermediärvorgänge bei der biologischen Kohlehydratspaltung, *Ergeb Enzymforsch*, 4: 208 (1935)

——— The Intermediary Reactions of Fermentation. *Nature*, 141: 855–858 (1938)

——— Intermediate Reactions of Fermentation. *Wallerstein Labs Communs*, 5 (No 16): 181–186 (1942)

——— New Chemical and Physical Tools for Investigating the Intermediary Metabolism of Carbohydrate, *Wallerstein Labs Communs*, 6 (No 17): 19–25 (1943)

——— Enzymatic Mechanisms of Fermentation. *Am Brewer*, January, 1943

——— The Origin of the Reaction of Harden and Young in Cell-free Alcoholic Fermentation, *Jour Biol Chem*, 157 (No 1) 105–119 (1945)

————: New Investigations in the Kinetics of Cell-free Alcoholic Fermentation, *Antonie Van Leeuwenhoek*, **12**: 140–144 (1947) (Jubilee Vol. A. J. Kluyver).

———— und W. Kiessling: Über das Auftreten und den Umsatz der α-Glycerinphosphorsäure bei der enzymatischen Kohlenhydratspaltung, *Biochem. Zeit.*, **264**: 40 (1933).

———— und ————: Über die phosphorylierten Zwischen-produkte und die letzten Phasen der alkoholischen Garung, *Biochem. Zeit.*, **267**: 313–348 (1933).

———— und ————: Über die Isolierung der isomeren Phosphoglycerinsäuren (Glycerinsäure-2-Phosphorsäure und Glycerinsäure-3-Phosphorsäure) aus Gäransätzen und ihr enzymatisches Gleichgewicht, *Biochem. Zeit.*, **276**: 239 (1935).

———— und ————: Über die enzymatische Umwandlung von Glycerinaldehydphosphosäure in Dioxacetonphosphorsäure, *Biochem. Zeit.*, **279**: 40 (1935).

———— und ————: Über die enzymatische Umsatz der synthetischen Phosphobrenztraubensäure (enol-Brenztraubensäure-phosphorsäure), CH$_2$=C(OH,PO$_3$)-COOH, *Biochem. Zeit.*, **280**: 99 (1935).

———— und ————: Die Umesterungsreaktion der Phosphobrenztraubensäure bei der alkoholischen Zuckergarung, *Biochem. Zeit.*, **281**: 249 (1935).

———— und ————: Über den Hauptweg der Milchsäurebildung in der Muskulatur, *Biochem. Zeit.*, **283**: 83 (1935).

———— und K. Lohmann: Über den Nachweis von Triosephosphorsäure als Zwischenprodukt bei der enzymatischen Kohlenhydratspaltung, *Naturwissenschaften*, **22**: 134 (1934).

———— ————: Über die enzymatische Gleichgewichts-reaktion zwischen Hexosediphosphorsäure und Dioxyacetonphosphorsäure. *Naturwissenschaften*, **22**: 220 (1934).

———— und ————: Über die enzymatische Gleichgewichts-reaktion zwischen Hexosediphosphorsäure und Dioxyacetonphosphorsäure, *Biochem. Zeit.*, **271**: 89 (1934).

———— und ————: Über die enzymatische Umwandlung von Phosphoglycerinsäure in Brenztraubensäure und Phosphorsäure, *Biochem. Zeit.*, **273**: 60 (1934).

———— ————: Über die enzymatische Gleichgewichts-reaktion zwischen Hexosediphosphorsäure und Dioxyacetonphosphorsäure. II. Die negative Wärmetönung der enzymatischen Spaltung, *Biochem. Zeit.*, **273**: 73 (1934).

———— und ————: Über die enzymatische Gleichgewichtsreaktion zwischen Hexosediphosphorsäure und Dioxyacetonphosphorsäure. III. Über Abfangen der Triosephosphorsäure mit Bisulfit und die Verbreitung des Ferments "Zymohexase" in den verschiedenen Zellarten, *Biochem. Zeit.*, **273**: 413 (1934).

———— ————: Über die enzymatische Gleichgewichtsreaktion zwischen Hexosediphosphorsäure und Dioxyacetonphosphorsäure. IV. (Berichtigung zur II. Mitteilung), *Biochem. Zeit.*, **275**: 430 (1935)

————, und P. Schuster: Über das Aldolase ein Kohlenstoffverknüpfendes Ferment   Aldolkondensation von Dioxyacetonphosphorsäure mit Acetaldehyd, *Biochem. Zeit.*, **286**: 301, 319 (1936).

———— and P. Oesper: The Mechanism of the Oxidative Reaction in Fermentation, *Jour Biol Chem*, **170** (No 1): 1–22 (1947)

————, P. Ohlmeyer, W. Gentner, und H. Meir-Leibnitz: Studium der Zwischenreaktionen der Glykolyse mit Hilfe von radioaktivem Phosphor, *Biochem. Zeit.*, **298**: 396 (1938)

————, ————, und W. Möhle: Über die Koppelung zwischen Oxydoreduktion und Phosphatveresterung bei der anaeroben Kohlenhydratspaltung, *Biochem. Zeit.*, **297**: 90 (1938)

——— und W. Schulz: Eine neue Bestimmungsmethode der Phosphoglycerinsäure, *Biochem. Zeit*, 297: 60: 1938.

Michaelis, L.: Chemistry of Alcoholic Fermentation, *Ind Eng Chem*, 27: 1037 (1935).

———, V. Moraques-Gonzales, and C V Smythe: Action of various dyestuffs on fermentation and phosphate synthesis in yeast extract, *Enzymologia*, 3: 242 (1937).

——— and C. V. Smythe: Influence of Certain Dyestuffs on Fermentation and Respiration of Yeast Extract, *Jour Biol. Chem*, 113: 717 (1936)

Myrbäck, K.: Die Co-Zymase und ihre Bestimmung, *Zeit physiol Chem*, 177: 158 (1928)

———: Co-Zymase, *Ergeb. Enzymforsch*, 2: 139 (1933).

———: Zur Kenntnis der Co-Zymase, V, *Zeit physiol Chem*, 233: 95 (1935).

——— und H von Euler: Garungs-Co-Enzym (Co-Zymase) der Hefe, IV, *Zeit. physiol Chem*, 138: 1 (1924)

——— und ———: Co-Zymase und die Aktivierung der Gärung Irischer Hefe durch Hefenextrakten, *Zeit physiol Chem*, 176: 238 (1928)

——— und B Örtenblad: Zur Kenntnis der Co-Zymase, IV, *Zeit physiol. Chem*, 233: 87 (1935)

Neuberg, C: Development of Chemistry of Fermentation, *Am. Brewer*, 75: 22–30 (May, 1942)

——— und A Hildesheimer: Über zuckerfreie Hefegärungen, I, *Biochem. Zeit*, 31: 170 (1911)

——— und J. Hirsch: Über den Verlauf der alkoholischen Gärung bei alkalischer Reaktion II Gärung mit lebender Hefe in alkalischen Lösungen, *Biochem Zeit*, 96: 175 (1919)

——— und ———: Wirkungsweise der Abfangmethode bei der Acetaldehyd-Glycerin-Spaltung des Zuckers, *Biochem Zeit*, 98: 141 (1919)

——— und M Kobel: Die desmolytische Bildung von Methylglyoxal durch Hefen-enzym, *Biochem. Zeit*, 203: 463 (1928)

——— und ———: Die Bildung von Brenztraubensaure als Durchgangsglied bei der alkoholischen Zuckerspaltung Ihre Isolierung als Hauptprodukt der Gärung, *Biochem Zeit*, 216: 493 (1929)

——— und E. Reinfurth: Die Festlegung der Aldehydstufe bei der alkoholischen Gärung. Ein experimenteller Beweis der Acetaldehyd-Brenztraubensäure-Theorie, *Biochem. Zeit*, 89: 365 (1918)

Nilsson, R : Einige Betrachtungen über den glykolytischen Kohlenhydratabbau, *Biochem. Zeit*, 258: 198 (1933)

——— und H. von Euler: Co-Zymase und Adenosintriphosphat, *Zeit physiol Chem*, 204: 201 (1932)

Ohlmeyer, P : Über die Gargeschwindigkeit der Hexosediphosphorsäure und ihre Umesterung mit Adenylsäure, *Biochem Zeit*, 283: 111 427 (1935)

———: Darstellung von reiner Dihydrocozymase in Substanz, *Biochem Zeit*, 297: 66 (1938)

Ostern, P, and A J Guthke Über die Darstellung der Phosphoglycerinsäure, *Zeit. physiol Chem*, 348: 155 (1937)

Parnas, J K : "Handbuch der Enzymologie," II, Leipzig, 1940

———, C. Lutwak-Mann, und T Mann Über die Verkettung der chemischen Umsetzungen in der alkoholischen Gärung, *Biochem Zeit*, 281: 168 (1935)

Robison, R A New Phosphoric Ester Produced by the Action of Yeast Juice on Hexoses, *Biochem Jour*, 16: 809 821 (1922)

———— and E. J KING: Hexosemonophosphoric Esters, *Biochem. Jour.*, **25**: 323 (1931).

SMYTHE, C. V.: Phosphoric Acid Esters from Yeast Extract, *Jour Biol. Chem.*, **117**: 135 (1937).

————: An Improved Method of Preparing Hexosemonophosphate from Yeast Extract, *Jour. Biol Chem.*, **118**: 619 (1937).

STEPHENSON, M : "Bacterial Metabolism," 2d ed., Longmans, Green & Company, New York, 1939

WARBURG, O , und W CHRISTIAN: Über ein neues Oxydationsferment und sein Absorptionsspektrum, *Biochem. Zeit ,* **254**: 438 (1932).

CHAPTER VI

BREWING

Brewing, or the production of malt beverages, is the name given to the combined processes of preparing beverages from infusions of grains that have undergone sprouting (malting), and the fermenting of the sugary solution by yeast, whereby a portion of the carbohydrate is changed to alcohol and carbon dioxide. It is an ancient industry and was probably invented by the Egyptians

The term "malt beverage," according to the Federal Alcohol Administration Act[1] of May, 1937,

. . . means a beverage made by the alcoholic fermentation of an infusion or decoction, or combination of both, in potable brewing water, of malted barley with hops, or their parts, or their products, and with or without other malted cereals, and with or without the addition of unmalted or prepared cereals, other carbohydrates or products prepared therefrom, and with or without the addition of carbon dioxide, and with or without other wholesome products suitable for human food consumption

Beer, ale, porter, and stout are examples of malt beverages.

Composition of Beers.—The substances found in a beer will depend largely upon the nature and quality of the raw materials, the treatment of the sprouted grain or malt used in mashing, and the character of the ensuing fermentation, but storage and finishing operations will likewise affect the final composition.

In a normal beer one may expect to find carbohydrates—such as dextrin, maltose, and glucose—and protein derivatives such as peptones, amino acids, and amides, these products arising mainly as the result of the action of the enzymes of the malt. Hops contribute bitter substances, resins, essential oil, and tannic acid, but a portion of some of these substances is lost during the subsequent brewing operations. As a result of the alcoholic fermentation, the sugars of the wort are converted, in part at least, to ethanol and carbon dioxide, with much smaller quantities of glycerol and acetic acid; while some of the amino acids are transformed to higher alcohols and acids, for example, succinic acid. Lactic acid may be added to the wort or may accumulate as the result of

the action of lactic acid bacteria. Salts are always found and usually traces of oil. The finished beer contains 85 to 92 per cent of water by volume. Thus a beer is not a simple beverage but one that is capable of wide variation in composition unless the conditions of brewing are very carefully regulated.

Table 35 gives the analyses of several types of beer.

TABLE 35 —ANALYSES OF SOME MALT BEVERAGES[1]

Variety	No. of analyses	Specific gravity	Water	Carbonic acid	Alcohol by weight	Extract	Nitrogenous substances	Sugar as maltose	Gum and dextrin	Acid as lactic	Glycerin	Ash	Phosphoric acid
Schenk	205	1.0114	91.11	0.107	3.36	5.34	0.74	0.05	3.11	0.156	0.12	0.204	0.035
Lager	238	1.0162	90.09	0.196	3.91	5.79	0.71	0.88	3.73	0.151	0.165	0.228	0.077
Export beer	109	1											
Bock	84	1											
Weiss bier	26	1											
Porter	40	1											
Ale	38	1											

[1] Reprinted by permission, from "Food Inspection and Analysis," 4th ed. by Leach and Winton, published by John Wiley & Sons, Inc., New York.

Extent of Manufacture.—Some idea of the magnitude of the industry may be gained by noting the following table, which gives the malt beverage production for the six leading states in the United States for the Government fiscal years 1936 and 1946. For each state, the number of breweries operated is also indicated.

During the fiscal year 1946, Missouri, New Jersey, and California, in the order named, followed the lead of the first five states listed in Table 36 and produced more than Michigan.

TABLE 36.—MALT-BEVERAGE PRODUCTION, FISCAL YEARS 1936 AND 1946[1]

State	Production in barrels[2]		Breweries operated	
	1936	1946	1936	1946
New York	8,598,031	13,464,043	70	44
Pennsylvania	6,010,171	9,558,788	112	63
Wisconsin	5,736,531	9,054,093	91	61
Illinois	3,578,180	5,968,439	61	39
Ohio	3,432,921	5,917,205	54	39
Michigan	3,249,355	4,126,082	52	24
Total, United States	51,812,062	84,977,700	732[*]	471

[1] U S Treas Dept. Annual Report of the Commissioner of Internal Revenue, 1936 and 1946
[2] The standard beer barrel holds 31 gal
[*] Number operated during any part of the year

Fig. 33 Brewery (Courtesy of Dr. L. V. Burton, Editor, *Food Industries*)

American Brewing Practice.—Beer produced in the United States is
distinctive for two principal reasons. First, the American public prefers
a sparkling clear, light, and very pale beer—one that will remain brilliant
when very cold or warm. Second, owing to the differences in the com-
position and properties of our malts and cereals, departure from European
procedures has become necessary.

The Brewing Process.—The essential steps in the manufacture of
beer include the preparation of malt, mashing, boiling the wort with
hops, fermentation, and finishing operations. Malt is prepared by soak-
ing selected barley in water, permitting it to germinate and drying it
under carefully regulated conditions. Malting is sometimes carried on
as a separate industry, but it is so closely associated with the brewing
industry that it may be regarded as an essential part of the whole brewing
process. In mashing, ground malt is mixed with heated water and
prepared malt adjuncts are usually added. The enzymes liberated by
the malt digest the starches, proteins, and some of the other substances
present in the mash. The soluble products are dissolved in the water of
the mash and form the sweet wort. The mash is filtered, and the
resultant clear wort is boiled with hops, strained, cooled, and pitched
(inoculated) with selected yeast. A fermentation ensues, which is
carried out at a low temperature. For lager beer the temperature
is slightly lower than for ale Following this fermentation, the beer is
stored to mature or age. During the finishing operations, the beer is
carbonated, cooled, racked, and pasteurized.

Although the principles employed in the production of beer and ale
are essentially similar there are minor differences that are carefully regu-
lated. A different treatment of malt is required both as to the length of
the germinating period and the kilning of the malt; a higher temperature
of fermentation is used, and a different type of yeast. Beer yeasts are
so-called "bottom" yeasts, since the fermentation proceeds vigorously
in the depths of the tanks and there is a great deposition of cells, while
ale is fermented by "top" yeasts that produce great masses of cells in
the foam that forms abundantly at the surface. Beer contains larger
amounts of unfermented carbohydrates, especially dextrins, while ale
has a higher percentage of alcohol This is in part due to the more
complete conversion of the starch to fermentable sugar in the malt used.

Malt.—The preparation of malt is described in Chap. III.

Malt Adjuncts.—In some countries, beer is prepared from malt, hops,
and water only, but in the United States malt adjuncts are employed in
addition, owing to the fact that the barleys used for the preparation of
malt in this country are richer in protein than the barleys used in Euro-

pean countries   A high nitrogen content is usually undesirable for it tends to produce a satiating and relatively unstable beer

Besides reducing the nitrogen content of the wort, malt adjuncts supply additional carbohydrate to be acted on by the excess of diastase present and to be fermented by the yeast, and they help to produce a beer that is less satiating, paler in color, and relatively more stable

Malt adjuncts include certain grains and grain products (cooked or uncooked), sugar and sirups, and miscellaneous other carbohydrate products. In normal times in this country, corn and corn products (corn grits, corn flakes, corn sugar), rice, sugar, and sirups are used as the principal malt adjuncts. With a shortage in malt or the common malt adjuncts other materials may be used as is shown in Tables 37 and 38.

TABLE 37.—KINDS AND QUANTITIES OF MATERIALS USED IN THE PRODUCTION OF FERMENTED MALT LIQUORS BY PRINCIPAL STATES DURING THE FISCAL YEAR 1947 [1]

State	Malt	Corn and corn products	Rice	Wheat	Barley	Sorghum grain
New York	384 823,535	118 587 540	14 850 300	290 890	2 465 320	10 052 225
Pennsylvania	265 300 089	65 454 118	3 031 090	80 200	4 697 577	14,791 983
Wisconsin	271 524,439	100 463 311	27 569 890		661 078	6 733 880
Missouri	187 334 730	21 614 475	81 942 351		250 900	2 547 700
Ohio	178 291,337	58 796 892	13 217 799	15 800	1 474 421	1 800 321
New Jersey	150 033 150	33 699 631	388 320		615 695	1 821 990
California	149 418 577	40 326 641	4 176 450			6 962 179
Total, United States	2 517 788 652	719 300 506	172 199 735	789 790	13 777 421	88 995 870

State	Soybeans and soybean products	Sugar and sirups	Hops and hop extracts	Cassava and cassava products	Potatoes and potato products	Other materials
New York	737 518	35 298 897	6 474 399	19 574 237	3 160 485	10 500
Pennsylvania	158 812	41 322 489	6 338 023	5 449 039	375 880	
Wisconsin	967 913	12 559 790	4 030 021	24 437 885	218 309	
Missouri	267 913	3 305 265	3 547 782	165 756	314	
Ohio	237 044	15 941 864	2 706 479	2 795 821	1 100	
New Jersey	156 820	18 270 996	2 743 066	2 985 607	175 109	
California	669 072	3 916 878	2 347 918	3 379 423	115 760	
Total, United States	4 885 115	218 378 826	40 506 917	107 629 992	6 657 927	70 717

[1] U S Treas Dept Annual Report of the Commissioner of Internal Revenue June 30 1947
[2] Quantities in pounds.

A mixture of 20 to 35 per cent of malt adjuncts and 65 to 80 per cent of malt is commonly used in the manufacture of beers in this country [1]

[1] POZES, M A, Ind Eng Chem, 26: 1127 (1931)

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Although the principles employed in the production of beer and ale are essentially similar there are minor differences that are carefully regulated. A different treatment of malt is required both as to the length of the germinating period and the kilning of the malt; a higher temperature of fermentation is used, and a different type of yeast. Beer yeasts are so-called "bottom" yeasts, since the fermentation proceeds vigorously in the depths of the tanks and there is a great deposition of cells, while ale is fermented by "top" yeasts that produce great masses of cells in the foam that forms abundantly at the surface. Beer contains larger amounts of unfermented carbohydrates, especially dextrins, while ale has a higher percentage of alcohol. This is in part due to the more complete conversion of the starch to fermentable sugar in the malt used.

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pean countries A high nitrogen content is usually undesirable for it tends to produce a satiating and relatively unstable beer.

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Wisconsin	271 524 459	100 463 311	27 369 800		664 078	6 753,880
Missouri	187 334 730	21 634 473	61 942 351		250 900	2 547 708
Ohio	178 291 337	58 796 892	13 717 790	15 800	1,474 421	1 800,321
New Jersey	150 033 150	53 089 631	586 320		615 695	5 821,091
California	149 418 977	40 326 641	4 176 450			8 962 170
Total, United States	2 313 788 652	710 300 506	172 199 731	789 700	13 777 421	88 995 670

State	Soybeans and soybean products	Sugar and sirups	Hops and hop extracts	Cassava and cassava products	Potatoes and potato products	Other materials
New York	737 538	35 299 897	6 474 339	19 354 217	3 160 465	10 500
Pennsylvania	156 812	41 322 489	4 339 023	3 449 979	375 800	
Wisconsin	967 913	12 559 790	4 030 021	24 437 885	218 300	
Missouri	78 112	3 305 265	3 547 782	165 756	394	
Ohio	237 944	15 941 864	2 706 479	2 791 821	1 100	
New Jersey	156 820	16 790 995	2 743 066	2 905 607	175 100	
California	669 072	3 958 878	2 317 918	3 359 421	155 760	
Total, United States	4 885 118	218 599 824	40 506 911	107 629 982	6 651 922	70 213

[1] U S Treas Dept Annual Report of the Commissioner of Internal Revenue June 30 1947
[2] Quantities in pounds.

A mixture of 20 to 35 per cent of malt adjuncts and 65 to 80 per cent of malt is commonly used in the manufacture of beers in this country.[1]

[1] POZEN, M A, Ind Eng Chem, 26: 1127 (1934)

In Table 37 are shown the kinds and quantities of materials used in the production of fermented malt liquors.

The quantities of materials used in the production of each barrel of fermented malt liquors are shown in Table 38. These are, of course, average figures based on nationwide production and are intended to convey only a broad picture of the malt-beverage industry. The materials used vary from plant to plant.

TABLE 38.—AVERAGE QUANTITIES OF MATERIALS USED IN THE PRODUCTION OF A BARREL OF FERMENTED MALT LIQUORS[1,2]

Material	Pounds per 31-gal. barrel			Percentage of total material		
	1937	1946	1947	1937	1946	1947
Malt	37 05	25 457	28 600	71.20	55.40	64.669
Corn and corn products	7 20	9 301	8 180	13 85	20 62	18 500
Rice	3 92	2 831	1.960	7.55	6.28	4.430
Wheat		0 565	0.0088		1 25	0 0199
Barley		1 878	0.157		4.15	0 3549
Sorghum grain		1 842	1 012	..	4.08	2 292
Soybeans and soybean products		0 079	0 0556	.	0.18	0 126
Sugar and sirups	3 22	2 664	2 490	6.19	5.90	5 630
Hops and hop extracts	0 63	0 442	0 461	1 21	0 98	1 042
Cassava and cassava products			1 225			2 770
Potatoes and potato products			0 0757	. .		0.171
Other materials		0 065	0 0008		0.15	0 0018
Total material per barrel	52 02	45 130	44 226	100 00	100 00	100 00
Total malt adjuncts[2]	14 34	19 231	15 165	27 59	42 62	34 289

[1] Calculated from statistics presented in the annual reports of the Commissioner of Internal Revenue, U.S Treas Dept, June 30, 1937, June 30, 1946, and June 30, 1947.
[2] Data are for fiscal years ending June 30
[3] Total materials less malt, hops, and hop extracts

Mashing.—The purpose of mashing is to digest and to dissolve as much as possible of the valuable portions of the malt or malt adjuncts.

The sweet wort that results from this enzyme process contains dextrins, maltose, other sugars, pentosans, protein degradation products, minerals, tannin, coloring matter, and other substances.

Mashing Methods.—There are two main methods for mashing: the infusion and decoction methods. Many modifications of these methods are used in practice.

THE INFUSION METHOD.—There are two infusion processes: one of these is the upward method; the other, the downward method. In the upward process, the malt is mixed (doughed in) with water at a tem-

perature of 38 to 50°C. This main mash is permitted to rest (protein rest period) for about 1 hr. at this temperature to favor the action of the proteolytic enzymes. The temperature is then raised to 65 to 70°C. by the doughing in of the cooked starchy malt adjuncts, which are at the boiling temperature. The mash is permitted to stand at this temperature for a few minutes for starch saccharification. The temperature is then brought to about 75°C., or a little above, for the destruction of the enzymes. The mash is filtered at this temperature.
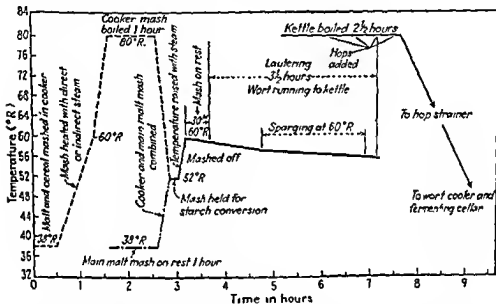
Fig 34.—Time-temperature chart of brewhouse operations. [Courtesy of Dr. R. Schwarz, Ind. Eng. Chem., 27: 1031 (1935).]

In the downward infusion process, the initial temperature of the mash water may be about 77°C. The added malt aids in cooling the water to approximately 70°C. A temperature of 65 to 70°C. is maintained, as in the upward infusion process, to permit saccharification. The final temperature of the mash is lower than the initial temperature. The downward infusion process is an English method.

THE DECOCTION METHOD.—In this method, the mash is mixed at a lower temperature, occasionally around 40°C., than is the case with infusion processes. The temperature of the mash is raised by steps until a final temperature of about 75°C has been obtained.

A portion of the initial mash, approximately one-third, is withdrawn, heated, and boiled for a short period of time and then returned to the main mash. The heated portion raises the temperature of this entire mash. The enzymes in the boiled portion have been destroyed, but the

The wort and spargings are placed in a brew kettle, usually made of copper, and the hops are added in the proportion of 0.55 to 0.9 lb. per standard beer barrel of 31 gal.

Boiling the Mash.—Wort containing hops is boiled for several reasons: to concentrate it, to sterilize it, to inactivate the enzymes, to extract soluble substances from the hops, to precipitate coagulated proteins and other substances, and to slightly caramelize the sugar. The addition of the spargings to the main wort dilutes it, making concentration desirable. The danger of the growth of undesirable microorganisms in the wort is lessened by sterilizing it and handling it under aseptic conditions. Inactivation of the enzymes aids in maintaining a wort of fairly constant composition for fermentation.

The substances extracted from hops include bitter acids and resins, essential oil, and tannin. The bitter acids and resins contribute to the palatefulness, colloidal stability, and head retention of the beer. The bitter acids are humulon or α-bitter acid $(C_{21}H_{30}O_5)$, and lupulon or β-acid $(C_{26}H_{38}O_4)$, respectively. By oxidation and polymerization the acids may be converted to soft resins. Both the acids and their corresponding resins possess antiseptic properties and impart characteristic flavors to beer. Humulon, however, possesses the strongest bitter flavor and the greatest antiseptic action. A third resin, of little value to the brewing industry, is the hard gamma resin.

A large proportion of the antiseptic action of hops is lost during the drying of the hops, their storage before use, boiling them with the wort, cooling, fermentation, and storage of the beer in barrels.[1]

Some flavor is imparted to beer from the essential oil of hops Since the essential oil is volatile in steam, most of it is lost during the boiling of the wort, unless the hops are added toward the end of the boiling process.

Tannins are extracted principally from the hops during boiling, but some are extracted from the malt. The tannins from barley possess an unpleasant taste. Hence their removal by reaction with the proteins of the wort, before the hops are added, is advantageous.

Tannin aids in the precipitation of some of the nitrogenous substances of the wort. Some of the tannin complexes precipitate out during boiling, but others[2] tend to become insoluble in the cold, giving rise to a chill haze, unless removed during subsequent cooling.

[1] WALKER, T. K., A Review of Ten Years' Research on the Antiseptic Constituents of Hops, *Jour. Inst. Brewing*, **38**: 198 (1932).

[2] LUERS, H., and C. ENDERS, Acidity and Protein Turbidities in Beer, *Compt rend. trav. lab. Carlsberg, Sér. chim.* **22**: 329 (1938).

Tannins are negatively charged. They react readily with positively charged proteins, forming complexes that become less soluble as the temperature decreases. Tannins react less readily with electrically neutral proteins, *i.e.*, proteins that are unstable.

Boiling converts some of the tannin of the hops into phlobaphene. Protein phlobaphene forms also during the boiling of the wort, and, being insoluble in the hot wort, it precipitates out. Oxygen accelerates protein-phlobaphene formation [1]

Agitation and circulation of the wort during boiling increases the amount of precipitation and hence the quantity of sludge formed. During cooling, agitation is also of much advantage in increasing the amount of precipitation. The greater the quantity of unstable compounds removed during boiling and cooling, the less likelihood there is of the formation of precipitates in the finished product.

After the wort has been boiled, it is filtered through a hop strainer to remove the hops and precipitated proteins. The wort may, or may not, be passed into a tank located above the cooler where settling may be permitted for 0.5 to 1 hr. Some cooling takes place in this tank. The wort is then cooled by passing it over or through coolers, preferably with considerable agitation.

During cooling, the wort becomes aerated and the rH* may increase from 12 or 13 to 16 or 17 [2]. Protein-tannin compounds precipitate out owing to their insolubility at the lower temperatures. A secondary precipitation is induced wherein proteins and hop resins are adsorbed on the surfaces of the protein-tannin compounds

The rate at which the wort is cooled between the temperatures of 60 and 21.1°C. has a direct bearing on protein-tannin precipitation,[3] brighter worts being secured through quicker cooling rates

Preventing contamination of the wort during cooling is essential

Fermentation. *Yeasts.*—Strains of *Saccharomyces cerevisiae* are commonly used in the manufacture of beer. The selection of an appropriate strain is a most important factor in determining the character of the beer

[1] Horkins, R. H., *Prevention Better Than Cure for Brewing Troubles, Food Industries*, 10: 74 (1938)

[2] Horkins and Krauss, "Biochemistry Applied to Malting and Brewing," D. Van Nostrand Company, Inc., New York, 1937

[3] Sippel, G. B., *Recent Advances in Brewing Technology, Food Research*, 3: 269 (1938).

* This is a term which represents the "negative logarithm of the hypothetical hydrogen pressure in equilibrium with the oxidation-reduction system" being studied M. Stephenson, "Bacterial Metabolism," 2d ed., Longmans, Green & Company, New York, 1939

This type of turbidity is likely to appear when the malt has been improperly dried in the kiln or when a barley with very high protein content is used. Warming the beer causes the turbidity to disappear.

Oxidation turbidity or haze is due in part to protein-tannin compounds. The presence of oxygen; the shaking of the beer during its transportation; the collisions of beer bottles, which impart supersonic and high-pitched sound vibrations; and sunlight affect the formation of oxidation haze. Saturation of the beer with carbon dioxide does much to prevent this turbidity.

Tannin-protein hazes appear also at low temperatures. In order to produce stable, chillproof beers, which will not become hazy or turbid when cold, the use of a small amount of a proteolytic enzyme preparation is advantageous. The enzymes are usually added after the fermentation although they may be added earlier. *The enzymes in an acid medium, such as is found in a beer, render the beer stable and chillproof.* Credit is due to Wallerstein[1] for this discovery.

Starch turbidities develop as a result of the improper conversion of starch during mashing. Lack of proper digestion at this time may be due to the use of a malt in which the diastase has been destroyed during the kilning. Sparging with water at a temperature much higher than 80°C. may also result in the production of turbidity. Amylases may be added to the storage vats to remove starch turbidity.

The presence of resin oil containing pitch may, rarely, cause turbidity, as may calcium oxalate. Proper filtration will prevent both types of turbidities.

Yeast turbidity may be due to lack of proper clarification during the secondary fermentation, which in turn is caused by an unsatisfactory wort. The use of chips, or krausening (the addition of beer in an active state of fermentation) is usually effective in correcting this type of turbidity. Wild yeasts, especially of the *Saccharomyces pastorianus* III species, produce turbidity. By excluding air and keeping the concentration of fermentable sugars in the beer low, growth of yeasts is inhibited. *A low rH, 12 or below, will also inhibit the growth of yeasts* according to De Clerck.[2] The use of pure cultures and proper sanitation of the plant should prevent the access and development of wild yeasts.

Among the turbidities caused by bacteria, those produced by sarcinae are most common, especially in bottom fermentations. Bacterial turbidities are less common than turbidities caused by yeasts and frequently will contain yeasts as well as bacteria. Aseptic technique, the use of

[1] Wallerstein, L., U.S. Patents 995,820 and 995,824, 1911.
[2] De Clerck, *Jour. Inst. Brewing,* **40:** 407 (1934).

pure cultures of yeasts, sanitation of the brewery, and a high content of hop antiseptics should prevent bacterial turbidities.

Faulty beer may be the result of the use of a low-grade raw material in the mash; the use of hops of poor quality, too much hops, or prolonged boiling of the hops; contact of the beer with iron, causing an inky taste; contact with tin; an unsuitable brewing water; young or green beer; or carbon dioxide deficiency; etc.

Beer Infection.—The term "beer infection" or "beer disease" is commonly used in describing the undesirable condition that occasionally exists in beers or ales as the result of the presence of microorganisms, chief among which are the bacteria. The microbial agents causing the symptoms may be designated as beer-infection microorganisms or beer-disease microorganisms.

The infection bacteria described in literature are included in five families: the *Pseudomonadaceae, Bacillaceae, Bacteriaceae, Micrococcaceae*, and *Lactobacteriaceae*. However, the bacteria of only three of these families, *Pseudomonadaceae, Bacteriaceae*, and *Lactobacteriaceae*, are of particular significance to the brewing industry. The actual number of genera involved is also small, as illustrated by Tosic,[1] who reviewed the subject of beer-infection organisms in 1915 and who reported that beer infections are caused by species of the following genera: *Acetobacter, Lactobacillus, Streptococcus, Flavobacterium*, and *Achromobacter*. There appears to be some doubt as to whether bacteria of the genera *Bacillus* and *Micrococcus* cause infections of malt beverages. The mere fact that sporeformers exist in the raw materials used in brewing is no indication that they are producers of infection

At this point it may be well to consider some of the conditions encountered in the brewing operation that have in general pronounced effects on bacteria and that, in fact, may determine whether or not an organism will survive or develop in the beer. These conditions are as follows:

1 The constituents of the wort are usually boiled for about 2 5 hr
2 The yeast fermentations are carried out at low temperatures
3 The pH values of the wort and beer are low
4 Hop antiseptics are present in the wort and beer
5 The beer is usually pasteurized
6 Anaerobic conditions usually prevail during fermentation and in the beer
7 Ethyl alcohol is present in the fermentation mash and in the beer

It is entirely conceivable that an unusually heat-resistant sporeformer, for example, strain of one of the species of the genera *Bacillus* or *Clos-*

tridium, might survive the long boiling period of 2 to 2.5 hr. to which the wort is subjected.

However, such bacteria are commonly Gram-positive and sensitive to hop antiseptics. A surviving sporeformer in order to cause infection would have to be tolerant of a relatively low pH, of anaerobic conditions, and of the alcohol normally found in a beer or ale. Thus, there are ample reasons why sporeformers may not be associated with beer infections.

Bacteria of the species of the genera *Acetobacter*, *Achromobacter*, *Flavobacterium*, *Lactobacillus*, and *Streptococcus* are destroyed within a relatively short time at temperatures well below the boiling point. Hence, if beverages contain these organisms, they have generally become infected during one or more of the operations following the boiling of the wort.

The species of the genus *Acetobacter* are rod-shaped, though involution forms may occur, Gram-negative, catalase positive (with the exception of *A. peroxidans*), aerobic, and grow usually in the range of 5 to 8 to 35 to 40°C. These bacteria produce acetic acid aerobically from ethyl alcohol and are tolerant of acid and of hop antiseptics in the amounts commonly found in beers. They may be responsible for the production of acidity or sourness in beers due to acetic acid. Two other species, *A. capsulatum* and *A. viscosum*, may produce ropiness in beer, while a third species, *A. turbidans*, may give rise to turbidity and sourness.[1,2] The latter organism apparently has the ability to develop when only a very small amount of oxygen is present.

The acetic acid bacteria, with the exception noted above, are dependent in their action upon the presence of oxygen. Consequently infections by species of the *Acetobacter* are limited to those cases where the fermented wort or beer is exposed to oxygen, as for example, in casks stored for a prolonged period, in empty beer barrels, and in the pitching yeast. *Control is exercised by excluding oxygen from the fermented product and in using pure cultures of yeasts.*

Two genera of the family *Lactobacteriaceae*, namely *Lactobacillus* and *Streptococcus*, contain species which may be causes of serious trouble. Bacteria of the genus *Lactobacillus* are rod-shaped, Gram-positive, catalase-negative, microaerophilic, and tolerant to acid. They may be tolerant to hop antiseptics or often adapt themselves to its presence. *L. pastorianus*,[3,4] synonyms of which are *Bacille des Bières Tournées* Pasteur and *Saccharobacillus pastorianus* Van Laer, gives rise to sourness in beers

[1] Cosbie, A. V. C., J. Tosic, and T. K. Walker, *Jour. Inst. Brew.*, 47: 382 (1941); 48: 82 (1942)

[2] Cosbie, A. V. C., J. Tosic, and T. K. Walker, *Jour. Inst. Brew.*, 49: 88 (1943)

[3] Shimwell, J. L., *Wallerstein Labs. Communs.*, 4 (No. 11): 41 (1941)

[4] Tosic, *loc. cit.*

and a silky type of turbidity. It may produce lactic, acetic, and formic acids, alcohol, and carbon dioxide from fermentable sugars and grow in the range of 11 to 37°C.

According to Shimwell,[1] *Lactobacillus pastorianus* is probably the most common defect-producing organism to be found in top fermentation breweries. Although difficult to remove entirely from a brewery, once it has gained entrance, the problem may be attacked in several ways. The plant should be kept clean, the yeast used for pitching should be pure, the residual fermentable extract should be kept at a minimum, the pH should be as low as possible consistent with the production of a quality product, the hop rate should be high at the time of racking, and finally the beer should be pasteurized, because *L. pastorianus* is destroyed by effective heat treatment

The streptococci causing trouble in beer are associated with such defects as "sarcina sickness," sourness, turbidity, and ropiness. Sarcina sickness is a disease of beer characterized by a honey-like odor. This particular odor is due to the production of diacetyl by the bacteria, which combined with the normal odor of the beer, produces the honey-like aroma (Refer to the section concerned with butter starters)

The species of bacteria concerned in the production of sarcina sickness, turbidity, sourness, and ropiness is *Streptococcus damnosus* (Claussen) Shimwell and Kirkpatrick. A synonym of this organism is *Pediococcus damnosus* Claussen. Shimwell[1] also lists as possible synonyms *P. perniciosus* Claussen, *P. cerevisiae* Balcke, and *P. sarcinaeformis* Reichard.

Streptococcus damnosus is nonsporeforming, Gram-positive, catalase-negative, anaerobic, tolerant to acid but with an optimum pH of 5 0 to 6.0.

Two varieties of *Streptococcus damnosus* have been described. These are *S. damnosus* var *viscosus* and *S. damnosus* var *pentosaceus* (Walters). The first of these varieties was isolated from Scotch ale by Claussen and is characterized by the production of slime in beers that are saturated with carbon dioxide. In flat beers, sarcina sickness is produced rather than slime.

Streptococcus damnosus var *pentosaceus* (originally named *S. tetragenus* var *pentosaceus*) was isolated from Australian beer by Walters.

The family *Bacteriaceae* contains two genera which are of considerable importance to the brewing microbiologists *Achromobacter* and *Flavobacterium* *A anaerobium* produces turbidity and off-colors in beers. *F proteus* is an infection of brewers' yeast.

Achromobacter anaerobium, a Gram-negative, nonsporeforming anaerobe, is tolerant of acids and hops. Actually it will grow at a pH of less than 4 and is not inhibited by the action of hop antiseptics in the con-

[1] Shimwell, *op cit*, pp 41 48

TABLE 40—A SUMMARY OF SOME INFORMATION CONCERNING THE DEFECTS OF BEER CAUSED BY BACTERIA[1]

Defect	Specific causative agent	Morphology and size (in microns)	Gram reaction	Catalase reaction	Relation to oxygen	Reaction to acid	Reaction to hops	Relation to temperature	Products
	Family—Pseudomonadaceae								
Sourness	*Acetobacter aceti*	0.4-0.8 by 1.0-2.0	Neg	Pos	Aerobic	Tolerant	Tolerant		Acetic acid
	A. acetosum	0.4-0.8 by 1.0	Neg	Pos	Aerobic	Tolerant	Tolerant	Range, 8-35°C	Acetic acid
	A. rancum	0.3-0.8 by 2.4-20	Neg	Pos	Aerobic	Tolerant	Tolerant	Range, 8-35°C	Acetic acid
	A. kutzingianum	Short, thick rods	Neg	Pos.	Aerobic	Tolerant	Tolerant	Range, 7-42°C.	Acetic acid
	A. linabers	Rods	Neg.	Pos.	Aerobic	Tolerant	Tolerant		Acetic acid
	A. mobile	Rods	Neg.	Pos.	Aerobic	Tolerant	Tolerant		Acetic acid
	A. oxydans	0.8-1.2 by 2.4-2.7	Neg.	Pos	Aerobic	Tolerant	Tolerant		Acetic acid
	A. pasteurianum	0.4-0.8 by 1.0	Neg	Pos	Aerobic	Tolerant	Tolerant	Range, 5-40°C	Acetic acid
	A. suboxydans	Short rods	Neg	Pos	Aerobic	Tolerant	Tolerant		Acetic acid
	A. xylinum	Short rods	Neg	Pos	Aerobic	Tolerant	Tolerant		Acetic acid
Ropiness	*A. capsulatum*	Coccoid rods 0.6-1.5	Neg	Pos.	Aerobic	Tolerant	Tolerant		Acetic acid
	A. viscosum		Neg.	Pos	Aerobic	Tolerant	Tolerant		Acetic acid
Sourness and turbidity	*A. turbidans*	Short rods at 25°C, involution forms at 40°C.	Neg	Pos.	Aerobic to microaerophilic	Tolerant	Tolerant	Grows in beers at 13°C, destroyed in 10 min. at 52°C.	Acetic acid
	Family—Bacteriaceae								
Turbidity, off-odors, and off-flavors	*Achromobacter anaerobium*	Rods 1-1.5 by 2-10	Neg.		Anaerobic	Tolerant	Tolerant	Destroyed in 5 min. or more at 60°C.	

Infection of brewers' yeast	Organism	Morphology	Gram	Motility	Oxygen relation (facultative anaerobe)	pH (no growth at pH 4·0; opt pH = 5·0)	Tolerance	Acid tolerance	Temperature (grown in beers at 18°C, destroyed in 5 min at 54°C)	Products
Infection of brewers' yeast	*Flavobacterium proteus*	0·4–1·2 by 1·5–4·0; Highly pleomorphic	Neg	Neg	Facultative anaerobe	No growth at pH 4·0; Opt pH = 5·0	Tolerant	Tolerant	Range 11–37°C	Lactic, acetic, and formic acids; alcohol, CO_2
	Family—*Lactobacteraceae*									
Sourness, sourness, wildness, turbidity, ropiness	*Lactobacillus pastorianus*	0·5–1·0 by 7–35	Pos	Neg	Microaerophile	May reduce pH to 3·8	Tolerant	Sensitive but may acquire resistance	Opt 21–25°C	Lactic and diacetyl
	Streptococcus damnosus	Cocci in pairs, tetrads, clumps. About 1	Pos	Neg	Anaerobe	May reduce pH to below 4	Tolerant	Sensitive but may acquire resistance		Diacetyl, acid, etc.
	S. damnosus var *mucosus*	Cocci in pairs, tetrads, chains	Pos	Neg	Anaerobe	Grows at pH 3·5		Sensitive but may acquire resistance	Grows at 15°C	Diacetyl, acid, etc.
	S. damnosus var *perbrevicus*	Cocci in pairs, tetrads, chains	Pos	Neg	Anaerobe			Sensitive but may acquire resistance		

[1] Information obtained from articles by Topaz, Shimwell, and others which are referred to in the references at the end of this chapter

[2] Data secured from Bergey's "Manual of Determinative Bacteriology" and other sources.

centrations used in breweries. **It grows only in "primed" beers but rapidly when the temperature is elevated.** Glucose and fructose but not maltose are utilized. A silky turbidity and odors of hydrogen sulphide (slight) and of apples are produced. Ethyl alcohol and carbon dioxide are formed. **The organism is destroyed by heat-treatment for 5 min or longer at 60°C.**

The infection of brewers' yeast, caused by *F. proteus*, has been described by Shimwell,[1] and Shimwell and Grimes.[2] Shimwell isolated the organism from "pitching" yeast. It is a Gram-negative, nonspore-forming, facultative anaerobe, which produces ethyl alcohol and acid and gives rise to a parsnip-like odor and flavor in both hopped and unhopped wort. Its activities, however, are restricted to the primary fermentation on account of its inability to grow at a pH of about 4.2. Since the pH of the wort may be 5.2 or more at the beginning of the fermentation, it may grow until the lowered pH inhibits further development.

Table 40 summarizes some information concerning the defects of beer caused by bacteria.

Definitions.

Lager beer is literally stored beer. The term "lager" is derived from "lagern," a German verb meaning "to store." According to this definition, all beer would be lager beer. Lager beer is produced by bottom fermentation and is rather high in alcohol and extract with a relatively low proportion of hops.

Bock beer is a heavy beer, dark in color and high in alcohol. It is brewed for consumption in the early spring

Ale is produced by top fermentation, is pale in color, tart in taste, high in alcohol and contains more hops than does beer.

Porter is a dark ale, high in extract and sweeter than the usual ale in taste. It is brewed from dark or black malt (malt roasted at a high temperature) to produce a wort of high extract. The flavor of hops is less distinct than that of normal ale.

Stout is a strong porter that is high in alcohol and extract. It is dark in color and possesses a sweet taste and strong flavor of malt. The hop flavor is more pronounced than that of porter.

Weiss bier, a beer made mainly from wheat, is produced by top fermentation It is rather light, possesses a marked flavor of malt and hop, is tart, and contains a large quantity of natural fermentation gas. It is likely to be turbid in appearance.

[1] Shimwell, J. L., *Jour. Inst Brew*, **42: 119 (1936)**
[2] Shimwell, J. L., and M. J. Grimes, *Jour. Inst Brew*, **42: 348 (1936).**

Cereal beverage is beer containing less than 0.5 per cent alcohol. It is sometimes known as "near beer"

Brewery Thermometers.—The brewer frequently uses the Reaumur thermometer instead of the Fahrenheit or Celsius (centigrade) scales. A comparison of a few points on the three scales is given in the following table:

TABLE 41.—A COMPARISON OF SOME POINTS ON THE REAUMUR, CENTIGRADE, AND FAHRENHEIT SCALES

Reaumur	Centigrade	Fahrenheit
−32	−40	−40
−16	−20	−4
0	0	32
4	5	41
8	10	50
16	20	68
32	40	104
48	60	140
52	65	149
56	70	158
80	100	212

It will be noticed that the Reaumur degree is equivalent to 1.25 centigrade degrees

Regulations.—Regulations concerning malt beverages are published by the Federal Alcohol Administration of the U S Treasury Department. The labeling and advertising of malt beverages, for example, are considered in Regulations 7 of the department

Additional Information.—Further information concerning brewing may be obtained by referring to the publications listed at the end of the chapter. The texts of Hopkins and Krause, Hind, and Vogel, Schwaiger, Leonhardt, and Merten will be found to be of special value. "Bottle Beer Quality," a record of 10 years of research at the Wallerstein Laboratories, is also of particular interest

Some Other Alcoholic Beverages.—Alcoholic beverages are consumed in every country of the world. In some countries the use of a particular beverage has been passed down from antiquity, for example, kvass in Russia, pulque in Mexico, taette in Scandinavia, and sorgho in Manchuria. A brief description of a few alcoholic beverages follows.

Kvass may be prepared by mixing equal parts of barley malt, rye malt, and rye flour, adding boiling water, stirring, permitting the mash to stand for several hours, adding more boiling water, and then inoculating

with yeast and permitting fermentation to take place. Peppermint is added to the fermented product for flavoring. This beverage is very common in Russia. Neighboring countries prepare similar drinks in which other starchy or saccharine materials may be used.

Pulque is prepared by allowing the sweet juice of agave (the century plant is a common species) to undergo a spontaneous fermentation, which is usually complete in about 1 day. Yeasts produce alcohol from the sugars. Bacteria are present, which cause a rapid spoilage of the beverage unless it is consumed shortly after its manufacture. Pulque resembles cider and has a flavor somewhat similar to that of sour milk. It is a very common beverage in Mexico.

Tacite is an alcoholic beverage prepared from milk. Yeasts cause the characteristic changes in flavor. The product has a pleasing acid taste

Sorgho is an alcoholic drink made from *Sorghum saccharatum.*

Sake is the widely used alcoholic beverage of the Japanese. It is a yellow rice wine containing 14 to 24 per cent of alcohol. There are various methods by which it is manufactured. Usually a *koji*, known as *sake-koji* or *tane-koji*, is prepared. Steamed rice is inoculated with the spores of *Aspergillus oryzae* and incubated at about 20°C. until the rice is well covered with mycelium. The *koji*, or starter, is mixed with steamed rice and some water and inoculated at a low temperature. Starch is converted to fermentable sugars. The thick liquid resultant from this enzyme hydrolysis and in which spontaneous fermentation usually takes place is known as *moto*. *Koji*, moto, and more water are usually mixed. An alcoholic fermentation ensues in which several yeasts may be active Saccharomyces sake, S. tokyo, and S. yeddo are some of the yeasts characteristic of sake.

Pombe is an alcoholic beverage made by permitting millet seed to sprout and undergo a conversion of the starch to sugars and by allowing a spontaneous fermentation of the saccharified starch in water.

Bili is a wine made from the tubercles of *Osbeckia grandiflora.* It is a West African drink.

Ginger beer is characterized by its distinctly acid nature, the ginger flavor, and the presence of a small amount of alcohol Carbon dioxide is evolved in considerable quantity. The raw materials are sugar (in 10 to 20 per cent concentrations) and pieces of ginger root. "Ginger-beer plant" is added to a solution of the sugar. In the ginger-beer plant are a yeast, *Saccharomyces pyriformis*, and a bacterium, *Bacterium vermiforme.* The yeast cells are entangled in the gelatinous sheaths of the bacteria A symbiotic relationship apparently exists, for both the yeast and bacterium function best when in each other's presence.

Some American Brewing Periodicals

American Brewer (monthly), A Schwarz Sons, New York, 1867–

American Brewers' Review (monthly), American Brewers' Review Co., Chicago, 1887–1937.

Brewers Bulletin (semiweekly), The Brewers Bulletin, Inc., Chicago, 1907–

Brewers Digest (formerly *Siebel Technical Review*) (monthly), Siebel Publishing Co., Chicago, 1925–

Brewers Journal (formerly *Western Brewer*) (monthly), H. S. Rich & Co., Chicago, 1876–

Brewery Age (monthly) Brewers Age Publishing Co., Chicago, 1932–

Modern Brewer (incorporated with *Brewer's Art*) (monthly), Modern Brewery, Inc., New York, 1920–

The West Coast Brewer (monthly), The West Coast Brewer, San Francisco, 1936–

ULRICH, C F Periodicals Directory, 1938, R R Bowker Co., New York, 1938.
(Contains a list of brewing periodicals.)

References on Brewing

ARNOLD, J. P., and F PENMAN "History of the Brewing Industry and Brewing Science in America," G L Peterson, Chicago, 1933

ATKIN, L., and P P GRAY: Influence of Wort Constituents on the Rate of Fermentation, *Wallerstein Labs Communs* 10 (No 31), 217–224 (1947)

BORUFF, C S: Industrial Wastes, Recovery of Fermentation Residues as Feeds, *Ind Eng Chem*, 39 (No. 5) 602 (1947)

CHRZASZCZ, T, and J JANICKI Recent Advances in the Fermentation Industries, *Chem and Ind* 55: 884 (1936)

CLARK, W M A Laboratory Mashing Apparatus, *Ind Eng Chem (Anal Ed)*, 11: 41 (1939)

COOPER, I M "References Ancient and Modern to the Literature on Beer and Ale," United Brewers Industrial Foundation, New York, 1937

COSPER, A J C Brewing, the Story of a National Industry, *Wallerstein Labs Communs*, 5 (No 15) 85–114 (1942)

DE CLERCK, J Papers concerned with oxidation-reduction in relation to brewing, *Wochschr Brau*, 51: 196, 201, 213, 378 (1934) *Jour Inst Brewing*, 40: 107, 117 (1934) *Am Brewer*, 68 (January, 1935) *Modern Brewer*, 18 (No 6) 38 (1937) *Modern Brewer*, 19 (No 1) 40 (1938)

EMSLIE, L E Uses Laboratory to Direct Process, Assures Quality, *Food Ind*, 11 (No 2) 76 (1939)

GRAY, P P, and J STONE Copper and Iron in Worts Yeast and Beer, *Wallerstein Labs Communs*, 5 (No 16) 193 199 (1942)

———, ——— and L ATKIN Systematic Study of the Influence of Oxidation on Beer Flavor, *Wallerstein Labs Communs*, 10 (No 31) 183 194 (1947)

GUILLIERMOND, A "The Yeasts" (translated and revised by F W Tanner), John Wiley & Sons, Inc New York 1920

HANES, C S Starch Structure and Enzyme Action, *New Phytologist*, 36: 101, 189 (1937)

HELM, E, and H TROLLE Experiments on Bottom Fermentation I Methodology, *Wallerstein Labs Communs*, 10 (No 30) 57 68 (1947)

HIND H L Pasteur to 1936 An Account of the Development of Science in Brewing, *Chem and Ind* 56 125 (1937)

————. "Brewing Science and Practice," Vol. I, "Brewing Materials," John Wiley & Sons, Inc, New York, 1938   (A second volume of this series is in preparation)

HOPKINS, R H · Prevention Better Than Cure for Brewing Troubles, Food Ind., 10: 74 (1938)

————. Enzymes in Malting and Brewing, Am. Brewer, 76 (No. 1): 34 (1943).

———— and C. B. KRAUSE: "Biochemistry Applied to Malting and Brewing," D. Van Nostrand Company, Inc., New York, 1937.

JORGENSEN, A · "Practical Management of Pure Yeast" (revised by Albert Hansen), Charles Griffin & Company, Ltd , London, 1936.

KOCH, J R , O. NELSON, and L. EHRNST: Viscometer for Routine Determination of Proteolytic Activity of Malts, Ind. Eng. Chem. (Anal. Ed.), 11: 35 (1939).

LAUFER, S.: The Colorimetric Determination of Oxidation-reduction Potential and Its Application to Brewing Practice, Am Brewer (December, 1935).

———— and R. SCHWARZ: "Yeast Fermentation and Pure Culture Systems," Schwarz Laboratories, Inc., New York, 1936

LEACH, A E, and A L. WINTON: "Food Inspection and Analysis," 4th ed , John Wiley & Sons, Inc., New York, 1920

LUERS, H The Significance of Oxygen in Brewing, Wochschr. Brau., 54 (No. 49): 389 (1937).

———— and C ENDERS: Acidity and Protein Turbidities in Beer, Compt. rend. trav lab Carlsberg, Sér chim, 22: 329 (1938).

MEES, R H "Onderzoekingen over de Biersarcina," Drukkerij Gebroeders Hoitsems, Groningen, 1934.

MENDLIK, F : Oxydations—Reduktionspotentiale im Bier, Wochschr. Brau, 51: 307 (1934). Am. Brewer, 68: (February, 1935).

NOWAK, C A : "Modern Brewing, A Practical Handbook of Contemporary Brewing Practice," 2d ed , Frank A Roth Co., St. Louis, 1934.

————. "Twelve Treatises on Practical Brewing," Modern Brewer, New York, 1935

NUGEY, A L "Brewing Formulas," Jersey Printing Co , Inc., Bayonne, N.J., 1937.

PERSONS, W M · "Beer and Brewing in America   An Economic Study," United Brewers Industrial Foundation, New York, 1937.

POZEN, M A · Enzymes in Brewing, Ind. Eng Chem., 26: 1127 (1934).

PYLER, E J. Yeast Propagation in the Modern Brewery, Brewers Digest, 14 (No 2): 25 (1939)

REICH, G T.: How to Convert Brewery to Industrial Alcohol Productions, Food Inds , 14 (No 5) 46 (1942).

SCHADE, A L . Origin and Activity of Enzymes during Malting, Communications on the Science and Practice of Brewing, p. 13, December, 1937

SCHWARZ, R Brewing Processes, Ind Eng Chem , 27: 1031 (1935)

SHIMWELL, J L Some Facts and Fallacies in Brewing Bacteriology, Jour. Inst. Brewing, 44 (No 12) (1938)

————: The Classification and Identification of Bacteria, with Special Reference to Beer Types, Jour Inst. Brewing, 46 (No. 6): 207 (1940).

———— The Lactic Acid Bacteria of Beer, Wallerstein Labs Communs., 4 (No. 11): 41–48 (1941)

————: The Gram Stain and Its Significance in Brewing Bacteriology, Wallerstein Labs. Communs . 8 (No 23) 23–33 (1945).

———— Yeast Contaminants. Brewers Digest, 22 (No. 4) · 45T (1947)

SIEBEL, F P , Jr Wartime Adjustments in Brewing Materials, Modern Brewery Age, 29 (No. 6): 29 (1943)

Siebel, Jr., F. P., and E. Singruen: Application of Oxidation-Reduction Potential to Brewing Control, *Ind. Eng. Chem.*, 27: 1012 (1935).

Singruen, E.: The History of American Brewing Literature, *Modern Brewer*, 19 (No. 4): 40 (1938).

Sippel, G. B.: Recent Advances in Brewing Technology, *Food Research*, 3: 269 (1938).

Trolle, B.: Experiments on Bottom Fermentation, II The Influence of Pitching Rate on Fermentation and on the Finished Beer, *Wallerstein Labs. Communs*, 10 (No. 30): 99–107 (1917)

United Brewers Industrial Foundation: "American Beer and Ale A Handbook of Facts and Figures," New York

U.S. Dept. of the Treasury, Federal Alcohol Administration. Federal Administration Act as in Effect on May 15, 1937

U.S. Treas. Dept., Annual Report of Commissioner of Internal Revenue for Fiscal Year 1936, ibid., 1937.

Vogel, E. H., Jr., F. H. Schwaiger, H. G. Leonhardt, and J. A. Merten "The Practical Brewer," Master Brewers Association of America, St. Louis, 1916

Walker, T. K.: A Review of Ten Years' Research on the Antiseptic Constituents of Hops, *Jour Inst Brewing*, 38: 198 (1932)

Wallerstein, J. S.: The Rôle of Hops in Brewing, *Wallerstein Labs. Communs*, 3 (No. 8): 45–54 (1940)

Wallerstein Laboratories *Communications on the Science and Practice of Brewing*, New York, December, 1937–

———; "Bottle Beer Quality," Wallerstein Laboratories, New York, 1917

Wilcox, J. K.: "A List of English, French, and German Periodicals on Brewing, Distillation, Wine and Other Alcoholic Beverages," The John Crerar Library, Chicago, 1933

Woon, L. D.: "Hops Their Production and Uses," The National Hops Company, Chicago, 1938

Some Patents

Clark, B. S.: Coating Composition for Sheet Metal Containers, U S Patent 2,167,638 Aug 1, 1933.

Glauitz, M., and H. Hahn: Beer Manufacturer U S Patent 1,898,017, Feb 21, 1933

Gray, P. P., and I Stone Beer and Method of Preparing Same, U S Patent 2,159,985, May 30, 1939.

Heimann, E.: Malt-enzyme Extraction in Beer Making, U S Patent 2 163,200, June 20, 1939

King, H J H: Malt House, U S Patent 2,150,769, Mar 14, 1939

Lindsay, R L. Process of Manufacturing Malt, U S Patent 2,137 111, Nov 15, 1938

Rach, C: Process for Making Hopped Beer Work, U S Patent 2,163 168, June 20, 1939

Schock, R. C Lauter Machine, U S Patent 2,150,009, Mar 7 1939

Walsh, J F., and W L Monson Process for Preparing a Brewing Adjunct, U S Patent 2,144,471, Jan 17, 1939

CHAPTER VII

WINE

Wine is the product made by the "normal alcoholic fermentation of the juice of sound, ripe grapes, and the usual cellar treatment."[1]

Classifications and Definitions.—Wines may be classified in many ways.

Dry wine is "wine in which the fermentation of the sugars is practically complete."[1] Most dry wines contain a small amount of sugar even though the quantity may be so slight as to escape detection by the sense of taste.

Sweet wine is "wine in which the alcoholic fermentation has been arrested."[1] Such wines contain sufficient sugar for taste perception. Wines may be fortified by the addition of brandy or wine spirits.

Fortified dry wine is "dry wine to which brandy has been added but which conforms in all other particulars to the standard of dry wine."[1]

Fortified sweet wine is "sweet wine to which wine spirits have been added."[1]

Sparkling wine is "wine in which the after part of the fermentation is completed in the bottle, the sediment being disgorged and its place supplied by wine or sugar liquor and/or dextrose liquor, and which contains, in 100 cc. (20°C.), not less than 0.12 g. of grape ash."[1] Such wine contains considerable carbon dioxide.

Wines may be red or white wines. A red wine is one containing the red coloring matter extracted from the skins of the grapes; a white wine is one "made from white grapes or the expressed fresh juice of other grapes."[2] In making a red wine, the skins and seeds are usually left with the must during fermentation.

"Modified wine, ameliorated wine, corrected wine is the product made by the alcoholic fermentation, with the usual cellar treatment of a mixture of the juice of sound, ripe grapes with sugar and/or dextrose, or a sirup containing not less than 65 per cent of the sugars, and in quantity not more than enough to raise the alcoholic strength after fermentation to 11 per cent by volume."[2]

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[1] U. S. Dept. of Agriculture, F. D. A., *Service and Regulatory Announcements, Food and Drug*, No. 2, Rev. 5, November, 1936
[2] *Ibid.*

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Raisin wine is the "product made by the alcoholic fermentation of an infusion of dried or evaporated grapes, or of a mixture of such infusion or of raisins with grape juice."[1]

Regions of Production.—A large part of the world's wine is produced in the countries located near the Mediterranean Sea France leads the world in the manufacture of wine, followed by Italy and Spain Portugal, Greece, the Balkan States, and Germany; Algeria and other regions of North Africa; Chile and Argentina; Australia, Canada, and the United States produce considerable quantities of wine.

There are three principal regions of wine production in the United States, represented by (1) California; (2) Louisiana, Arkansas, and Missouri; and (3) New York, Ohio, New Jersey, and Michigan [2]

Imports.—The imports of sparkling and still wines with their respective values for the years indicated are shown in Table 42.

TABLE 42.—AMOUNT AND VALUE OF IMPORTS OF SPARKLING AND STILL WINES[1][2]

	Quantity, thousands of gallons			Value, thousands of dollars		
	1931	1936	1917	1931	1936	1917
Sparkling wines	395	502	242	2,971	3 441	2,167
Still wines	3 163	3 134	4 919	10 212	8 021	6,643

[1] *U S Dept of Commerce* Statistical Abstract of the United States, 1937
[2] The 1947 data were obtained from *U S Bur Census, Foreign Trade Report* 110 U S Imports for Consumption of Merchandise 1947

During the calendar year 1917, the United States imported 181,974 gal. of Champagne, worth $1,610,373, chiefly from France and Italy; and 858,281 gal. of Vermouth, worth $2,197,035, principally from Italy, France, and Argentina. In addition, large quantities of grape wine were imported, particularly from Europe and South America

Production Statistics.—Table 43 gives the production of still wine and the number of bonded wineries in the seven leading states and the United States during the fiscal years 1937 and 1916

A perusal of this partial table indicates that California produces most of the still wine manufactured in the United States. New York follows California as the second most important state

Sparkling wine production by eight leading states during the fiscal years 1937 and 1916 is as indicated in Table 44

[1] *Ibid.*
[2] GORE, H E , Notes on Wine Manufacture in the United States, *U.S. Dept of Agr*, mimeographed sheets, 1936

TABLE 43.—PRODUCTION OF STILL WINE AND NUMBER OF BONDED WINERIES IN LEADING STATES AND IN THE UNITED STATES, FISCAL YEARS 1937 AND 1946[1]

State	Production, wine gallons[2]		Bonded wineries[3]	
	1937	1946	1937	1946
California	115,338,166	365,008,818	630	413
New York	3,147,822	4,650,147	123	113
Washington	956,860	3,094,395	35	23
Oregon		2,873,479	.....	22
Michigan	479,609	925,263	11	11
Ohio	755,175	752,539	130	102
Georgia	334,815	600,866	12	4
Total, United States	122,015,241	370,935,981	1,206	880

[1] U S Treas Dept , Annual Report of the Commissioner of Internal Revenue, 1937 and 1946
[2] Includes distilling materials (substandard wines produced with excessive water or residue materials).
[3] Number operated during any part of the year.

TABLE 44 —PRODUCTION OF SPARKLING WINE, FISCAL YEARS 1937 AND 1946[1]

State	Production, half-pint units	
	1937	1946[2]
New York	4,652,321	19,276,246
California	1,531,358	13,664,356
New Jersey	1,502,366	3,918,110
Missouri	984,022	1,447,738
Ohio	736,705	1,677,353
Michigan	171,745	432,996
Washington	41,312	
Pennsylvania	2,696	
Wisconsin	.	152,589
Total, United States	9,622,525	40,569,388

[1] U S Treas Dept , Annual Report of the Commissioner of Internal Revenue, 1937 and 1946
[2] Includes production of 661,386 units of artificially carbonated wines

Chemical Composition of Wines.—In Table 45 are shown the chemical analyses of some wines of American origin made by wine makers receiving awards at the Paris Exposition during the year 1900. The data presented in the table were compiled and computed from the analyses given by Wiley For further details, the reader is referred to the publications of Amerine (1947), Amerine and Joslyn (1910), Joslyn and Amerine (1941), and Amerine and Winkler (1944)

Volatile Acids.—Acetic and propionic acids are the volatile acids found in sound wines Acetic acid is the principal volatile acid of young wines,

TABLE 15.—CHEMICAL ANALYSIS OF AMERICAN WINES RECEIVING AWARDS AT PARIS IN 1900[1]

(Grams per 100 cubic centimeters)

	Sparkling wines			Dry white wines			Sweet white wines			Dry red wines			Sweet red wines		
	Maximum	Minimum	Average	Maximum	Minimum	Average	Maximum	Minimum	Average	Maximum	Minimum	Average	Maximum	Minimum	Average
Specific gravity at 15.5°C	1.0129	0.9910	1.0045	0.9993	0.9901	0.9917	1.0404	0.9908	1.0296	0.9926	0.9903	0.9943	1.0522	1.0107	1.0276
Alcohol by volume per cent	13.20	11.05	13.22	14.75	10.60	12.45	21.55	11.60	18.38	15.40	10.10	12.64	22.05	13.70	19.30
Alcohol	12.90	9.03	10.43	14.01	8.40	9.98	17.10	9.21	14.58	12.22	8.51	10.00	17.50	10.87	15.71
Glycerol	0.7270	0.2701	0.4171	1.0110	0.3152	0.5709	0.7350	0.0483	0.3025	0.9696	0.3340	0.635	0.7400	0.2036	0.5089
Glycerol-alcohol ratio															
Extract	8.25	1.75	3.40	2.51	1.55	1.99	2.83		13.80	3.21	1.77	2.57	10.71	7.57	13.72
Ash	0.290	0.114	0.133	0.270	0.107	0.166	0.263	0.091	0.203	0.393	0.133	0.247	0.374	0.234	0.311
Total acids	0.782	0.601	0.715	0.713	0.435	0.586	0.805	0.100	0.412	0.901	0.414	0.649	0.826	0.307	0.502
Fixed acids	0.715	0.414	0.506	0.550	0.328	0.458	0.528	0.116	0.300	0.690	0.346	0.707	0.568	0.257	0.472
Volatile acids	0.094	0.019	0.049	0.093	0.001	0.091	0.222	0.029	0.092	0.246	0.071	0.128	0.253	0.090	0.122
Total tartaric acid	0.257	0.105	0.171	0.122	0.009	0.109	0.290	0.038	0.142	0.252	0.063	0.163	0.145	0.025	0.078
Free tartaric acid	0.147	0.004	0.062	0.155	0.000	0.060	0.1470	0.000		0.090	0.000	0.000	0.000	0.000	0.000
Polarization	−13.3	−1.8	−3.4	−1.1	−9.1	−6.7	+5.7	0.0		−16.1	−0.1	−7.7	−19.4	−7.7	−16.4
...	5.54	0.23	3.09	1.74	0.031	0.114	1.919	1.777	11.8	0.280	0.043	0.141	16.60	0.254	10.26
Reducing sugars	0.618	0.070	0.231	0.137	0.009	0.112	0.243	0.026		0.395	0.011	0.170	0.160	0.031	0.032
Protein	0.124	0.014	0.033	0.13	0.041	0.047	0.053	0.007	0.014	0.133	0.010	0.070	0.063	0.018	0.024
Potassium sulphate	0.091	0.012	0.017	0.009	0.010	0.022	0.079	0.008	0.071	0.044		0.029	0.068	0.018	0.043
Phosphoric acid	0.0456			0.0075	0.0001	0.0017	0.0036								
Free sulphurous acid	0.004	0.0010	0.0074	0.0255		0.0003	0.0188	0.0011	0.0045	0.0188	0.0011	0.0041	0.0047		
Total sulphurous acid	0.008	0.009	0.015	0.0530	0.0173	0.038	0.0662	0.0213	0.0363	0.039	0.0213	0.0316	0.2207	0.0516	0.0662
Tannin															

[1] Massey, H. —, American Wines at the Paris Exposition of 1900 Their Composition and Character, U S Dept Agr Bur Chem, Bull 72 1902

but old wines contain traces of propionic acid in addition.[1] Formic acid is usually found in diseased wines, together with acetic acid.

Fixed Acids.—Tartaric, malic, and tannic acids are constituents of the must and are, therefore, present in the wine in varying proportions. Much of the tartaric acid is removed during the manufacture of wine, especially during the fining process.[2]

Citric acid[3] is found in some grapes and in some wines. Succinic acid is produced in very small quantities during yeast fermentation. Phosphoric acid is a natural constituent of wine.

Wine Manufacture.—Although the fundamentals of wine making are similar in all three of the principal regions in the United States, methods vary to some extent in the different regions owing to the varieties of grapes used, the climate, and other factors. For example, a lower temperature may be used in the pasteurization of wines produced in the eastern portion of the United States on account of the greater acidity of the wine.

In view of the magnitude of the industry in California, special attention will be devoted to discussing wine making as it is practiced there, illustrating with a description of the production of red wine.

The reader desirous of securing additional knowledge of wine making will find numerous references at the end of this chapter, which should prove to be both interesting and instructive.

The Manufacture of Red Wine. *Outline of the Process.*—Selected grapes of the proper maturity are crushed and stemmed; treated with sulphur dioxide, or a sulphite, or pasteurized; and inoculated with a starter containing a pure culture of yeast. After a short fermentation period the wine is drawn off, placed in storage tanks for further fermentation, racked, stored for aging, clarified, and packaged.

Details of the Process. 1. THE GRAPE.—The production of a fine wine may be regarded as commencing with the selection of the best variety of grape for use in its manufacture. Bioletti[4] recommends the following varieties of grapes for the production of red wine: Beclan, Blue Portuguese, Cabernet Sauvignon, Carignane, Gros Verdot, Merlot, Mondeuse, Petite Sirah, Serine, Tannat, and Zinfandel for the coast counties of California; and Barbera, Grenache, Gros Mansenc, Lagrain, Pagadebito, Refosco, St. Macaire and Valdepeñas for the interior valleys of California.

[1] MORRIS, M M., Volatile Acids of Wine, *Ind. Eng Chem*, **27**, 1250 (1935).

[2] WOODMAN, A G., "Food Analysis," 3d ed., McGraw-Hill Book Company, Inc., New York, 1931

[3] WINTON, A L, and K B. WINTON, "The Structure and Composition of Foods," Vol. II, John Wiley & Sons, Inc., New York, 1935.

[4] BIOLETTI, F T, *Calif Agr Ext. Circ. 30*, Revised, 1934.

Amerine and Winkler[1] recommend the varieties shown in Table 45a for regions in California. The relative value of some white and red varieties of grapes for table and dessert wines is demonstrated in Table 45b

The quality of the grapes of a given variety will depend upon the conditions under which they are grown—soil, climate, and other conditions.

Grapes should be gathered at the proper stage of maturity   In order to determine the degree of maturity, representative bunches of grapes are picked, and the Balling degree of their juice is determined   A reading of 21 to 23° Balling is usually given by the juice of the grapes when they are at the optimum stage of maturity[2]

2. HANDLING THE GRAPES —In gathering the grapes and transporting them to the winery, the prime purpose should be to have them arrive in the very best condition possible   The grapes should be picked with care, placed in clean containers, and protected from deterioration   Careful supervision of the handling of grapes is essential

3 CRUSHING THE GRAPES.—Grapes are crushed and stemmed by machine. The chemical composition of the metal used in the construction of this machinery and other equipment about the winery is important. Iron and steel are used in some wineries but are undesirable for they may cause clouding of the wine, forming so-called "ferric casse" The tin and copper dissolved from bronze by grape juice, if sufficient in quantity, may cause flavor and color losses during the aging process Stainless steel, nickel or inconel should be used in preference to iron, ordinary steel, and many bronzes

If the grapes are not picked when cool, it is desirable to permit them to cool overnight before they are crushed.

4 TREATMENT BEFORE FERMENTATION —Grapes contain on their surfaces a varied flora of microorganisms—molds, yeasts, and bacteria It is quite possible that the juice of crushed grapes will produce a good wine without any special precautions, but a wine manufacturer cannot afford to gamble in respect to the quality of his wine   He can do much to ensure the quality of his final product by destroying or inhibiting the development of the microorganisms found on the grapes and by the use of starters containing pure cultures of the specific yeast desired

Sulphur dioxide or sulphites destroy or inhibit the growth of many undesirable types of microorganisms—acetic acid bacteria, wild yeasts, and molds—with a minimum amount of injury to the true wine yeast Usually 2 to 6 oz [1] or twice the quantity of potassium metabisulphite, are added per ton of crushed grapes, the quantity used depending on the

[1] AMERINE, M A, and A J WINKLER, *Hilgardia* 15 (No 6) 493 (1944)
[2] JOSLYN, M A, and W V CRUESS, *Calif Agr Ext Circ* 88, November 1931
[3] *Ibid*

TABLE 45a.—Recommended Varieties of Grapes for Regions in California[1,2]

Region 1 (less than 2,500 degree-days)[3]	Region 2 (2,501 to 3,000 degree-days)[3]	Region 3 (3,001 to 3,500 degree-days)[3]	Region 4 (3,501 to 4,000 degree-days)[3]	Region 5 (4,001 or more degree-days)[3]
White Wines				
High-quality dry table wines: White Riesling, Chardonnay, Sauvignon blanc	High-quality dry table wines: Pinot blanc, White Riesling, Sauvignon blanc. Standard, special, or blending dry table wines: Semillon, Sylvaner, Folle blanche	Natural sweet wines: Semillon, Sauvignon blanc, Muscat blanc. Standard, special, or blending wines: French Colombard, Pinot blanc	Standard-quality wines: French Colombard, Muscatels, Malvasia bianca, Muscat Canelli, Orange Muscat. Dessert wines: Mission, Palomino, Verdelho	Muscatels, Malvasia bianca, Orange Muscat. Dessert wines: Palomino, Mission, Grenache
Red Wines				
High-quality table wines: Cabernet Sauvignon, Pinot noir. Standard or blending wines: Gamay, Grenache	High-quality table wines: Cabernet Sauvignon. Standard, special, or blending wines: Grenache, Petite Sirah, Refosco	Standard, special, or blending table wines: Barbera, Carignane, Refosco. Dessert wine: Trousseau	Standard table wines: Barbera. Bulk-quality table wines: Carignane, Refosco. Dessert wines: Tinta Madeira, Trousseau, Muscatel, Aleatico	Dessert wines: Trousseau, Tinta Madeira. Special or blending wines: Salvador

See bottom of next page for footnotes.

TABLE 45b.—RELATIVE VALUE OF WHITE AND RED VARIETIES OF GRAPES FOR TABLE AND DESSERT WINES[1]

Table wines			Dessert wines		
Varieties	Relative value	Productivity	Varieties	Relative value	Productivity
White			White		
Chardonnay	92	Low	Palomino	82	High
White Riesling	88	Low	Muscat Canelli	82	Low medium
Semillon	85	Medium	Orange Muscat	77	High
Sylvaner	85	Low medium	Grey Riesling	76	High
Pinot blanc	83	Low	Malvasia bianca	73	Medium
Red			Red		
Cabernet Sauvignon	95	Low	Tinta Madeira	81	High medium
Pinot noir	94	Low	Mission	79	High
Grenache	82	High	Trousseau	77	High
Gnignolino	79	Medium	Grenache	77	High
Barbera	78	High medium	Zinfandel	73	High medium
			Aleatico	73	Medium

[1] Information obtained from M. A. Amerine and A. J. Winkler. Composition and Quality of Musts and Wines of California Grapes *Hilgardia* 15 (No. 6) 493–673 (1944). For information on additional varieties of lower relative value refer to article by Amerine and Winkler.

condition of the grapes—their maturity, the degree of contamination with molds, the temperature of the crushed product, and other factors. The largest quantities are used when the grapes are overripe, moldy, or relatively warm

[1] Information obtained from article by M. A. Amerine and A. J. Winkler *Hilgardia* 15 (No. 6), 493–673 (1944)

[2] By this term is meant the summation of heat above 50° F. for the period of April to October inclusive. For example if the mean temperature for July was 75°F. the summation would be (75 − 50) 31 = 775 degree-days

[3] Region 1 Napa and Oakville in Napa County Hollister and San Juan Bautista in San Benito County, Woodside in San Mateo County Mission San Jose in Alameda County Saratoga in Santa Clara County Bonny Doon and Vinehill districts in Santa Cruz County and Guerneville, Santa Rosa and Sonoma in Sonoma County

Region 2 Soledad in Monterey County Rutherford, St. Helena, and Rising Mountain in Napa County Santa Barbara in Santa Barbara County Asti in Sonoma Vineyard Evergreen (Santa Isabel) district, and San Ysidro in Santa Clara County and Glen Ellen in Sonoma County

Region 3 Livermore and Pleasanton in Alameda County Calistoga (Utah) and Hopland in Mendocino County Calistoga in Napa County Alpine in San Diego County Templeton in San Luis Obispo County Lomm Prieto in Santa Cruz County and Alexander Valley Asti, and Cloverdale in Sonoma County

Region 4 Gilroy in San Bernardino County Escondido in San Diego County Cucamonga, Escalon, Lockeford, Lodi, and Manteca in San Joaquin County Cordelia in Solano County Ceres, Hughson, and Vernalis in Stanislaus County Ukiah in Mendocino County and Davis in Yolo County

Region 5 Fresno and Sanger in Fresno County Madera in Madera County Arena and Livingston in Merced County and Tivola in Tulare County

Quoted from M. A. Amerine and A. J. Winkler Composition and Quality of Musts and Wines of California Grapes *Hilgardia* 15 (No. 6) 493–673 (1944)
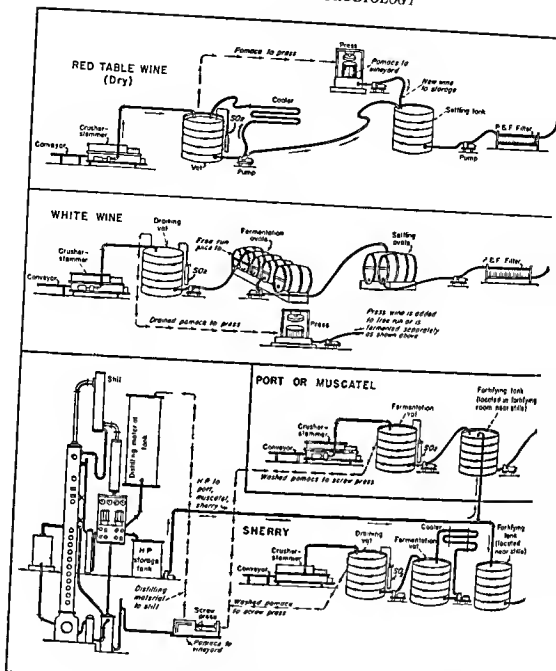
Fig. 35A.—Flow diagram of wine manufacture   [Courtesy of W. V.

Pasteurization may be used in place of sulphites but is not usually considered to be so desirable.

5. THE FERMENTATION.—The selection of a yeast, the nutrient substances in the must (grape juice), the concentration of the sugar, the acidity, the oxygen supply, and the temperature are factors that must be supervised in respect to fermentation.
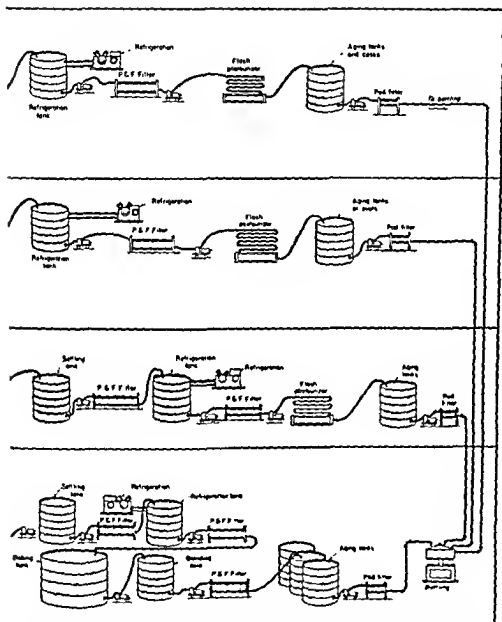
Saccharomyces ellipsoideus is the yeast used for the fermentation of must. Selected strains, such as the Burgundy or Tokay strains, are frequently used. Many strains, bearing different names, are known.

A starter is prepared from a pure culture of the selected yeast. Pasteurized must is used as the culture medium in preparing the starter, the magnitude of which should represent 2 to 5 per cent of the volume of the crushed grapes being inoculated.

It is usually unnecessary to add any substances for the nutrition of the yeast since the crushed grapes are an adequate source of supply. On rare occasions ammonium sulphate or phosphates may be added.

Joslyn and Cruess[1] state that the optimum concentration of sugar is 22° Balling. The use of much higher concentrations of sugar favors the production of more than 13 per cent of alcohol by volume. Since alcohol tends to inhibit the fermentation when present in concentrations of 13 to 15 per cent by volume, a maximum of 13 per cent is usually desirable. The concentration that actually inhibits fermentation depends in part on the temperature of fermentation, the tolerance of the yeast for alcohol decreasing with increasing temperature. The approximate concentration of the alcohol that will be produced in the wine can be predetermined by multiplying the Balling reading of the must by 0.575.

It is permissible to reduce the concentration of sugar in must by the addition of water.[2] Another practice is to mix the juice with the high sugar concentration with a juice of low sugar concentration. Occasionally sugar may be added to must.

Grapes that have been permitted to become too mature are frequently of low acidity. Fruit acid—tartaric, citric, or malic acids—may be added to restore the normal acidity.

A large supply of oxygen is essential for the rapid multiplication of yeast cells and the starting of the fermentation, as stated under yeast manufacture (Chap. IX), while the later stage characterized by alcohol and carbon dioxide production rather than growth proceeds best under nearly anaerobic condition.

Approximately 6 hr. after treating the crushed grapes with sulphur dioxide or sulphite, the starter is added. Thereafter the contents of the tank are mixed, or stirred, at least twice a day, except during the main fermentation, to facilitate aeration, temperature equalization, and the extraction of color and tannin. Normally a "cap" forms on the surface of the fermentation vat, which contains grape skins, pieces of stem, seeds and other suspended matter. To mix the contents of the tank, one may punch down the cap or pump juice from the bottom of the vat over the surface of the must.

The amount of aeration produced by mixing the contents of the tank is determined by the effectiveness of the procedure and by the frequency at which the operation is repeated. Provided that the fermentation is slow at the beginning, or near the end of the incubation period, the supply of oxygen may be increased by more frequent mixing of the contents of

[1] Ibid.

[2] Cruess, W. V., "The Principles and Practice of Wine Making," The Avi Publishing Co, Inc, New York, 1934

the vat. However, the must should not be overaerated during fermentation, for overaeration is likely to produce a wine of inferior quality, especially insofar as color and flavor are concerned

Fermentation should be carried out at carefully controlled temperatures. The finest wines are produced usually at temperatures below 85°F. (29.4°C.). The development of bouquet and aroma are favored by maintaining the fermenting must at low temperatures, around 70 to 75°F. (21.1 to 23.9°C.), for example A temperature range of 70 to 90°F. (21.1 to 32.2°C.) is satisfactory When the temperature rises to 85°F. (29.4°C.) or, at the most, to 90°F. (32.2°C.), the mash should be artificially cooled. Temperatures above 95°F. (35°C.) are considered unsafe, while the fermentation is inhibited usually at temperatures of 97 to 100°F. (36.1 to 37.8°C.) Fermentations cease at a temperature of 105°F. (40.5°C.)[1] generally. Undesirable bacteria develop at the higher temperatures. Accordingly the quality of the wine is impaired Obviously, at too low temperatures, the fermentation is too slow to be practical.

During the fermentation, records of the temperature and the Balling degree should be made at least twice a day, one set of observations being recorded on the side of the fermentation vat in order that the progress of the wine may be followed

After 3 to 5 days of active fermentation, sufficient tannin and a maximum of color have been extracted from the skin of the grape Extraction is facilitated by the agitation of pomace (skin, seeds, and pieces of stems) during fermentation, by the ethyl alcohol produced from the grape sugar, the heat of fermentation, and the mechanical breaking up of the skin

The wine maker decides when the color and tannin content are satisfactory and then draws off the wine to separate it from the pomace He does not wait for all the sugar to be fermented At the time of drawing off the wine, the Balling reading may be 0 to 1 It is not considered advisable to mix the wine drawn off ("free-run wine") with that expressed from the pomace, for the latter is of lower quality.

6 FURTHER FERMENTATION — The free-run wine is placed in closed storage tanks, equipped with bungs that allow the excess carbon dioxide to escape An atmosphere of carbon dioxide over the wine tends to inhibit the development of acetic acid bacteria and other aerobic types of microorganisms The fermentable sugar is usually consumed in 7 to 11 days at a temperature of 70 to 85°F (21 1 to 29 1°C)

If the after fermentation becomes sluggish before the sugar is utilized, the yeast may be activated by pumping over the wine

7 RACKING By racking is meant the drawing off of the wine from the lees or sediment Potassium bitartrate (KHC₄H₄O₆, ₁₆, cream of

[1] *Ibid*

tartar, is found in the lees. This substance is less soluble in alcohol than in water and precipitates out more rapidly at low temperatures.

Wine is racked to facilitate its clearing and to prevent undesirable flavors from being extracted from the old yeast.

8. STORAGE AND AGING.—Two important changes take place during storage and aging: clearing of the wine and the development of flavor

In a new wine there are present substances which, if not removed, will produce a sediment and probably cloudiness. These substances include tartrates, certain proteins, and other matter. Naturally these substances would be removed by racking and filtration during a somewhat long storage and aging process, but the modern trend is to hasten the removal of these substances by methods that involve flash pasteurization (to precipitate certain proteins), cooling to room temperature and then to 24 to 27°F. (−4.44 to −2.78°C.), and holding at the latter temperature for a few days. Filtration is carried out in the cold. Since the acid content of the wine is frequently reduced by the foregoing rapid process it is customary to adjust the acidity with citric or tartaric acids, the former acid being preferred. The wine is placed in tanks for aging.

Wine storage tanks are generally constructed of white oak or redwood, white oak being the better of the two The tanks are completely filled with wine and sealed to prevent the access of large quantities of oxygen, which would favor the growth of acetic acid bacteria and *Mycoderma vini* (wine flowers). Some wine is always lost through evaporation. Consequently, the tanks should be inspected regularly and kept filled with wine. Periodically the wine is racked. During racking and filling, especially, carbon dioxide is lost while some oxygen is absorbed. A small amount of oxygen accumulates in the headspace over the wine.

Flavor, which is due to a combination of taste and odor, is developed in wine as a result of oxidative changes and ester formation.

Aging proceeds slowly until oxygen becomes available in small quantities. It is inhibited by the presence of large quantities of carbon dioxide, by sulphur dioxide, and by the exclusion of air. New wines stored in airtight bottles do not age properly.

Alcohols, aldehydes, tannins, and other substances present in the wine are oxidizable. Alcohols may be converted to aldehyde and subsequently to acids by oxidation. Aldehydes form acetals with alcohol.

Combinations of alcohols with acids give rise to esters, which are important in the production of aroma or bouquet. Although opinions differ concerning the importance of esters, it is recognized that the nature of volatile esters is of greater significance than the quantity.[1] The esters of acetic acid contribute much to the flavor and bouquet of wine.

[1] NELSON, E. K., *Food Research*, 2: 221 (1937).

The time required for aging varies with the type of wine and the conditions. A dry wine may require at least 2 years. Some fine wines are aged for 5 or more years.

9. CLARIFICATION.—Wines may clear naturally over a period of time, but resort is frequently made to the use of finings followed by filtration, heating, refrigeration, or combinations of the foregoing. Fining agents, which include such substances as casein, gelatin and tannin, bentonite (of Wyoming origin, if possible), isinglass (fish protein from the sturgeon), white of egg, and Spanish clay, are mixed with the wine carefully according to direction, or preferably after laboratory tests have been carried out with small portions of the wine and the fining agents. The improper use of some of the fining agents may, in itself, be a cause of clouding of the wine.

Filtration is carried out with filter aids. For further details the reader is referred to "The Principles and Practice of Wine Making" by Cruess and to the more recent publications on this subject [1]

10. PACKAGING.—The clarified wine is placed in oak barrels for bulk sale and in bottles or in cans for unit sale.

Bottles of small and medium size may be pasteurized for 30 min at 140°F.[2]

Defects of Wine.—Defects of wine may be caused by microorganisms, in which case they are known as diseases, or by other agencies. The diseases of wine are of two general types: those caused by aerobic microorganisms, and those caused by facultative anaerobes or anaerobes.

Diseases Caused by Aerobic Microorganisms —The aerobic diseases of wines are caused principally by mycodermas and acetic acid bacteria. These microorganisms grow well in wine in the presence of oxygen. They cause no trouble if the wine is carefully supervised during its manufacture and storage. They are most likely to become active during the fermentation of must, if the cap is not punched down frequently, and during the storage of wine, if the containers are not kept properly filled and sealed.

Mycoderma vini (wine flowers) forms a film over the surface of wine and attacks the extract, the alcohol, and occasionally the organic acids.

Acetic acid bacteria will produce vinegar from wine in the presence of oxygen, unless they are destroyed or prevented from growing. Their activities are discussed in the chapter on vinegar.

Diseases Caused by Facultative Anaerobes or Anaerobes TOURNE DISEASE —The term "tourne" is considered to signify either the organism causing the disease or the condition produced in wine by large numbers of

[1] SAYWELL, L. G. *Ind. Eng. Chem.*, 27: 1245 (1935)
[2] CRUESS, W. V. *Fruit Products Jour.*, 15: 40 (1935)

these bacteria.[1]   Tourne is considered to be the most serious disease of wines and one of the most common.[2]

The organism is an anaerobic bacterium, which occurs as long, slender rods.   It may be found in red or white wines, or in fortified wines with an alcohol content of 20 per cent or greater.   It grows best, however, if the alcohol concentration is not too great.   Sugar and other nutrient substances favor its growth.   It is likely to develop in wines "stuck" due to high temperatures.   It is inhibited slightly by tannin but very strongly by sulphur dioxide and metabisulphites.

Tourne is indicated[2] by increasing volatile acidity, by decreasing fixed acidity, by a "silky" type of cloudiness, and, when the condition has progressed well, by an odor and taste that is termed "mousey."

Tourne may be detected by a microscopic examination of the sediment obtained by centrifuging a sample of the wine, or by analyses of the wine for volatile acids.   If the maximum amount of volatile acid permitted in wine by law—0.14 for red wine or 0.12 for white wine—has been produced or exceeded, then there is good evidence that the wine may be infected with tourne.   Taste may also be of some assistance in its detection.

The judicious application of sulphur dioxide, 75 p.p.m., to wines, or pasteurization; the use of a high degree of cleanliness about the plant, sterilization of equipment with steam when necessary; and rigid laboratory control should lower the incidence, or prevent, tourne disease of wine

A pasteurization of bottled wine at a temperature of 145°F. for 30 min is very effective in preventing tourne.

Once wine has been infected by tourne, it should be made brilliantly clear by filtration with selected infusorial earths, or by clarification with bentonite followed by passage through germproof filters[4] Sulphur dioxide, or its equivalent of metabisulphite, should then be added to the wine in such quantity that its concentration will be maintained at 75 p.p.m., or greater.   All equipment that has been infected should be treated with live steam or a suitable disinfectant to destroy the source of infection.

LACTOBACILLUS HILGARDII.—Spoilage of some dry wines in California has been caused by *Lactobacillus Hilgardii*,[5] a nonmotile, nonsporulating rod, which produces lactic and acetic acids.   A silky cloudiness is produced, while the flavor is affected and becomes somewhat "mousey."

[1] CRUESS, W. V., The Tourne Disease of Wine, *Fruit Products Jour.*, 14: 198 (1935)

[2] JOSLYN and CRUESS, *loc. cit.*

[3] JOSLYN and CRUESS, *loc. cit.*

[4] CRUESS, W. V., *Fruit Products Jour.*, 14: 198 (1935).

[5] DOUGLAS, H. C., and W. V. CRUESS, *Food Research*, 10: 113 (1936)

MANNITOL (MANNITE) FORMING BACTERIA.—Bacteria that produce volatile acid, lactic acid, and mannitol ($C_6H_{14}O_6$) from glucose grow well at temperatures above 100°F. Their growth is inhibited by keeping the temperature of fermentation well below this point, by the use of sulphur dioxide, and by an increase in the acid content of the wine due to the addition of citric or tartaric acid.[1]

SLIME FORMING BACTERIA.—Infected wine may become slimy and cloudy. Slime formation occurs chiefly in white wines, usually young wines in closed containers. The occurrence of slime-forming bacteria in wine is not common and may be prevented by adding tannin to wines low in this substance and by use of sulphur dioxide or metabisulphite.

OTHER BACTERIAL DISEASES.—Cocci, which may be inhibited by SO_2 or destroyed by pasteurization, may cause cloudiness in white wines.

Bitter wines may be caused by the growth of butyric acid bacteria, while a sour wine may result from the growth of lactic acid bacteria.

Defects Not Caused by Microorganisms.—Defects in wines may be caused by metals, enzymes, and the improper use of certain fining agents

Iron is a cause of clouding in wines Two different types of defects are produced by iron: one is known as black, blue, or ferric casse; the other as white casse.

Ferric casse is indicated by the appearance of a gray to gray-blue sediment in the wine and by clouding The defect is found in bottled white wines, especially Iron forms a precipitate with the tannin and coloring matter of red wine Only a few parts of iron in a million parts of wine will produce ferric casse Oxygen facilitates the formation of ferric ions from ferrous ions

Ferric casse may be prevented by using equipment constructed of the proper types of metal and inhibited by 0 1 per cent citric acid.

The defect may be treated in one of several manners In one method,[2] the iron is oxidized to the ferric stage by aeration. Tannin to the amount of 0 05 per cent is added Clarification with casein and bentonite follow. In a *second method*, the iron is oxidized by aeration, tannin is added, and then gelatin. Settling is permitted, which is followed by racking, filtration, and acidification with citric acid. In a third method, tartaric acid is added to the wine, and the wine is then refrigerated. Cream of tartar and iron salts are precipitated

White casse is also caused by an excess of iron The precipitate is due in part to iron phosphate It occurs in white wine. Treatment is as outlined above for ferric casse

Oxidase casse is uncommon It is caused by an enzyme, oxidase, produced by certain molds which causes white wines to become brown

[1] JOSLYN and CRUESS, *loc cit*
[2] SAYWELL, L G, *Ind Eng Chem* 26 379 (1934)

and the color to be precipitated in red wines. Sulphur dioxide inhibits this oxidase, while pasteurization at 180°F. destroys it.

Other Defects.—Tin, tin salts, copper, aldehydes, or excessive quantities of gelatin may cause clouding of the wine. For a further discussion of this subject the reader is referred to the publications of Cruess and other workers.

Coatings of Wine Tanks.—Concrete tanks are used for the fermentation of must and the storage of wine, but they must be lined to prevent an undue amount of calcium from being dissolved by the wine. Steel tanks should be lined to prevent the solution of iron

According to Cruess[1] and his associates one effective method for coating concrete is to treat the concrete first with a 0.5 per cent, then a 25 per cent, solution of tartaric acid. An insoluble calcium tartrate forms over the surface of the tank.

A mixture of 25 per cent Gilsonite and 75 per cent paraffin produces a protective coating for concrete and steel. Such linings are easily applied and not expensive.

Bass-Hueter black enamel gives good results[1] with both steel and concrete. A coating of paraffin over the enamel gives even better protection.

Standards of Identity for Wine.[2]—The Federal Alcohol Administration has set up standards of identity for wine, the text of which follows:

Article II　Sec. 20　*Application of standards.*—The standards of identity for the several classes and types of wine set forth herein shall be applicable to all regulations and permits issued under the act. Whenever any term for which a standard of identity has been established herein is used in any such regulation or permit, such term shall have the meaning assigned to it by such standard of identity

Sec. 21.　*The standards of identity.*—Standards of identity for the several classes and types of wine set forth herein shall be as follows.

Class 1.　*Grape wine.*—(a) "Grape wine" is wine produced by the normal alcoholic fermentation of the juice of sound, ripe grapes (including restored or unrestored pure condensed grape must), with or without the addition, after fermentation, of pure condensed grape must, and with or without added fortifying grape spirits or alcohol, but without other addition or abstraction except as may occur in cellar treatment, *Provided,* That the product may be ameliorated before, during, or after fermentation by either of the following methods.

(1) By adding, separately or in combination, dry sugar, or such an amount of sugar and water solution as will not increase the volume of the resulting product more than 35 per cent. but in no event shall any product so ameliorated have an

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[1] Cruess, W. V., T Scott, H B Smith, and L. M. Cash, *Food Research,* 2: 385 (1937).

[2] U S Dept of the Treasury, Federal Alcohol Administration, Regulations 4, Amendment 2, Washington, Aug. 22, 1938

alcoholic content, derived by fermentation, of more than 13 per cent by volume, or a natural acid content, if water has been added, of less than 5 parts per thousand, or an unfermented residual sugar content, derived from added sugar, of more than 11 per cent by weight.

(2) By adding, separately or in combination, not more than 11 per cent by weight of dry sugar, or not more than 10 per cent by weight of water.

The maximum volatile acidity, calculated as acetic acid and exclusive of sulphur dioxide, shall not be, for natural red wine, more than 0 14 gram, and for other grape wine, more than 0.12 gram, per 100 cubic centimeters (20°C.).

(b) "Red wine" is grape wine which contains the red coloring matter of the skins, juice, or pulp of grapes

. (c) "White wine" is grape wine which does not contain the red coloring matter of the skins, juice, or pulp of grapes.

(d) "Light wine" (including "light grape wine," "light red wine," and "light white wine") is grape wine having an alcoholic content not in excess of 14 per cent by volume.

(e) "Natural grape wine" (including "natural red wine" and "natural white wine") is grape wine containing no fortifying grape spirits or added alcohol

(f) "Angelica," "madeira," "muscatel," and "port" are types of grape wine containing fortifying grape spirits or added alcohol, having the taste, aroma, and characteristics generally attributed to these products, and an alcoholic content of not less than 18 per cent by volume

(g) "Sherry" is a type of grape wine containing fortifying grape spirits or added alcohol, having the taste, aroma, and characteristics generally attributed to this product, and an alcoholic content of not less than 17 per cent by volume.

(h) "Light port" and "light sherry" are types of grape wine containing fortifying grape spirits or added alcohol, having the taste, aroma, and characteristics generally attributed to "port" and "sherry," respectively, and an alcoholic content of more than 14 per cent by volume

Class 2   *Sparkling grape wine* —(a) "Sparkling grape wine" (including "sparkling wine," "sparkling red wine," and "sparkling white wine") is grape wine made effervescent with carbon dioxide resulting solely from the secondary fermentation of the wine within a closed container, tank, or bottle.

(b) "Champagne" is a type of sparkling light white wine which derives its effervescence solely from the secondary fermentation of the wine within glass containers of not greater than one gallon capacity, and which possesses the taste, aroma, and other characteristics attributed to champagne as made in the champagne district of France

(c) A sparkling light white wine having the taste, aroma, and characteristics generally attributed to champagne but not otherwise conforming to the standard for "champagne" may, in addition to but not in lieu of the class designation "sparkling wine," be further designated as champagne style or champagne type or "American (or New York State, California, etc) champagne bulk process" all the words in any such further designation shall be equally conspicuous and shall appear in direct conjunction with and in lettering approximately one-half the size of the words sparkling wine

Class 3. *Carbonated grape wine.*—(a) "Carbonated grape wine" (including "carbonated wine," "carbonated red wine," and "carbonated white wine") is grape wine made effervescent with carbon dioxide other than that resulting solely from the secondary fermentation of the wine within a closed container, tank, or bottle.

Class 4. *Citrus wine.*—(a) "Citrus wine" or "citrus fruit wine" is wine produced by the normal alcoholic fermentation of the juice of sound, ripe fruit (including restored or unrestored pure condensed citrus must), with or without the addition, after fermentation, of pure condensed citrus must, and with or without added fortifying citrus spirits or alcohol, but without other addition or abstraction except as may occur in cellar treatment, *Provided,* That the product may be ameliorated before, during, or after fermentation by adding, separately or in combination, dry sugar, or such an amount of sugar and water solution as will not increase the volume of the resulting product more than 35 per cent, but in no event shall any product so ameliorated have an alcoholic content, derived by fermentation, of more than 13 per cent by volume, or a natural acid content, if water has been added, of less than 5 parts per thousand, or an unfermented residual sugar content, derived from added sugar, of more than 11 per cent by weight.

The maximum volatile acidity, calculated as acetic acid and exclusive of sulphur dioxide, shall not be, for natural citrus wine, more than 0.14 gram, and for other citrus wine, more than 0.12 gram, per 100 cubic centimeters (20°C.)

(b) "Light citrus wine" or "light citrus fruit wine" is citrus wine having an alcoholic content not in excess of 14 per cent by volume.

(c) "Natural citrus wine" or "natural citrus fruit wine" is citrus wine containing no fortifying citrus spirits or added alcohol.

(d) Citrus wine derived wholly (except for sugar, water, or added alcohol) from one kind of citrus fruit, shall be designated by the word "wine" qualified by the name of such citrus fruit, *e g,* "orange wine," "grapefruit wine." Citrus wine not derived wholly from one kind of citrus fruit shall be designated as "citrus wine" or "citrus fruit wine" qualified by a truthful and adequate statement of composition appearing in direct conjunction therewith. Citrus wine rendered effervescent by carbon dioxide resulting solely from the secondary fermentation of the wine within a closed container, tank, or bottle shall be further designated as "sparkling"; and citrus wine rendered effervescent by carbon dioxide otherwise derived shall be further designated as "carbonated."

Class 5. *Fruit wine.*—(o) "Fruit wine" is wine (other than grape wine or citrus wine) produced by the normal alcoholic fermentation of the juice of sound, ripe fruit (including restored or unrestored pure condensed fruit must), with or without the addition, after fermentation, of pure condensed fruit must, and with or without added fortifying fruit spirits or alcohol, but without other addition or abstraction except as may occur in cellar treatment, *Provided,* That the product may be ameliorated before, during, or after fermentation by adding, separately or in combination, dry sugar, or such an amount of sugar and water solution as will not increase the volume of the resulting product more than 35 per cent, but in no event shall any product so ameliorated have an alcoholic content, derived

by fermentation, of more than 13 per cent by volume, or a natural acid content, if water has been added, of less than 5 parts per thousand, or an unfermented residual sugar content, derived from added sugar, of more than 11 per cent by weight

The maximum volatile acidity, calculated as acetic acid and exclusive of sulphur dioxide, shall not be, for natural fruit wine, more than 0 14 gram, and for other fruit wine, more than 0 12 gram, per 100 cubic centimeters (20°C.).

(b) "Berry wine" is fruit wine produced from berries

(c) "Light fruit wine" is fruit wine having an alcoholic content not in excess of 14 per cent by volume

(d) "Natural fruit wine" is fruit wine containing no fortifying fruit spirits or added alcohol

(e) Fruit wine derived wholly (except for sugar, water, or added alcohol) from one kind of fruit shall be designated by the word "wine" qualified by the name of such fruit, e g , "peach wine," "blackberry wine." Fruit wine not derived wholly from one kind of fruit shall be designated as "fruit wine" or "berry wine," as the case may be, qualified by a truthful and adequate statement of composition appearing in direct conjunction therewith. Fruit wines which are derived wholly (except for sugar, water, or added alcohol) from apples or pears may be designated as "cider" and "perry," respectively, and shall be so designated if lacking in vinous taste, aroma, and characteristics. Fruit wine rendered effervescent by carbon dioxide resulting solely from the secondary fermentation of the wine within a closed container, tank, or bottle shall be further designated as "sparkling"; and fruit wine rendered effervescent by carbon dioxide otherwise derived shall be further designated as "carbonated "

Class 6. *Wine from other agricultural products.*—(a) Wine of this class is wine (other than grape wine, citrus wine, or fruit wine) made by the normal alcoholic fermentation of sound fermentable agricultural products, either fresh or dried, or of the restored or unrestored pure condensed must thereof, with the addition before or during fermentation of a volume of water not greater than the minimum necessary to correct natural moisture deficiencies in such products, with or without the addition, after fermentation, of pure condensed must, and with or without added alcohol or such other fortifying spirits as will not alter the character of the product, but without other addition or abstraction except as may occur in cellar treatment, *Provided,* That the product may be ameliorated before, during, or after fermentation by adding, separately or in combination, dry sugar, or such an amount of sugar and water solution as will not increase the volume of the resulting product more than 35 per cent, but in no event shall any product so ameliorated have an alcoholic content, derived from fermentation, of more than 13 per cent by volume, or a natural acid content, if water has been added, of less than 5 parts per thousand, or an unfermented residual sugar content, derived from added sugar, of more than 11 per cent by weight.

The maximum volatile acidity, calculated as acetic acid and exclusive of sulphur dioxide, shall not be, for natural wine of this class, more than 0.14 gram, and for other wine of this class, more than 0.12 gram, per 100 cubic centimeters (20°C)

(b) "Light" wine of this class is wine having an alcoholic content not in excess of 14 per cent by volume.

(c) "Natural" wine of this class is wine containing no fortifying spirits or added alcohol.

(d) "Raisin wine" is wine of this class made from dried grapes.

(e) "Sake" is wine of this class produced from rice in accordance with the commonly accepted method of manufacture of such product.

(f) Wine of this class derived wholly (except for sugar, water, or added alcohol) from one kind of agricultural product shall, except in the case of "sake," be designated by the word "wine" qualified by the name of such agricultural product, e.g., "honey wine," "raisin wine," "dried blackberry wine." Wine of this class not derived wholly from one kind of agricultural product shall be designated as "wine" qualified by a truthful and adequate statement of composition appearing in direct conjunction therewith. Wine of this class rendered effervescent by carbon dioxide resulting solely from the secondary fermentation of wine within a closed container, tank, or bottle shall be further designated as "sparkling", and wine of this class rendered effervescent by carbon dioxide otherwise derived shall be further designated as "carbonated."

Class 7. *Vermouth.*—(a) "Vermouth" is a compound having an alcoholic content of not less than 15 per cent by volume, made by the mixture of extracts from macerated aromatic flavoring materials with grape wine containing fortifying grape spirits or added alcohol, and manufactured in such a manner that the product possesses the taste, aroma, and characteristics generally attributed to vermouth.

Class 8. *Imitation, concentrate, and substandard wine.*—(a) "Imitation wine" shall bear as a part of its designation the word "imitation," and shall include:

(1) Any wine containing synthetic materials.

(2) Any wine made from a mixture of water with residue remaining after thorough pressing of grapes, fruit, or other agricultural products.

(3) Any class or type of wine the taste, aroma, color, or other characteristics of which have been acquired in whole or in part, by treatment with methods or materials of any kind, if the taste, aroma, color, or other characteristics of normal wines of such class or type are acquired without such treatment.

(b) "Concentrate wine" shall bear as a part of its designation the word "concentrate," and shall include any wine made from must concentrated at any time to more than 80° (Balling)

(c) "Substandard wine" shall bear as a part of its designation the word "substandard," and shall include:

(1) Any wine having a volatile acidity in excess of the maximum prescribed therefor in this article.

(2) Any wine for which no maximum volatile acidity is prescribed in this article, having a volatile acidity, calculated as acetic acid and exclusive of sulphur dioxide, in excess of 0.14 gram per 100 cubic centimeters (20°C.).

(3) Any wine for which a standard of identity is prescribed in this article, which, through disease, decomposition, or otherwise, fails to have the com-

position, color, and clean vinous taste and aroma of normal wines conforming to such standard.

Sec. 22. *Blends, cellar treatment, alteration of class or type*—(*a*) If the class or type of any wine shall be altered, and if the product as so altered does not fall within any other class or type either specified in this article or known to the trade, then such wine shall, unless otherwise specified in this section, be designated with a truthful and adequate statement of composition in accordance with section 34, article III, of these regulations.

(*b*) Alteration of class or type shall be deemed to result from any of the following occurring before, during, or after production:

(1) Treatment of any class or type of wine with substances foreign to such wine which remain therein, *Provided*, That the presence in finished wine of not more than 350 parts per million (not more than 70 of such parts being in a free state) of total sulphur dioxide, or sulphites expressed as sulphur dioxide, shall not be precluded under this paragraph.

(2) Treatment of any class or type of wine with substances not foreign to such wine but which remain therein in larger quantities than are naturally and normally present in other wines of the same class or type not so treated.

(3) Treatment of any class or type of wine with methods or materials of any kind to such an extent or in such manner as to affect the basic composition of the wine so treated by altering any of its characteristic elements.

(4) Blending of wine of one class with wine of another class or the blending of wines of different types within the same class.

(5) Treatment of any class or type of wine for which a standard of identity is prescribed in this article with sugar or water in excess of the quantities specifically authorized by such standard, *Provided*, That where such wine is derived exclusively from fruit or other agricultural products the normal acidity of which is 20 parts or more per thousand, and such wine has been manufactured in accordance with the standard of identity therefor in all respects except that the volume of the product has been increased more than 35 per cent, but not more than 60 per cent, by the addition of sugar and water solution for the sole purpose of correcting natural deficiencies due to such acidity, the class or type shall not be deemed to be altered but there shall be stated, as a part of the class or type designation, the phrase "made with over 35 per cent sugar solution."

(*c*) Nothing in this section shall preclude the treatment of wine of any class or type in the manner hereinafter specified, provided such treatment does not result in the alteration of the class or type of the wine under the provisions of paragraph (*b*) of this section.

(1) Treatment with filtering equipment, and with fining or sterilizing agents

(2) Treatment with pasteurization at the minimum temperature and for the minimum period necessary to accomplish practical stabilization, but not for the purpose of shortening the normal maturation period.

(3) Treatment with refrigeration at the maximum temperature and for the minimum period necessary to accomplish practical stabilization, but not for the purpose of shortening the normal maturation period

(4) Treatment with methods and materials to the minimum extent neces-

sary to correct cloudiness, precipitation, or abnormal color, odor, or flavor developing in wine.

(5) Treatment with constituents naturally present in the kind of fruit or other agricultural product from which the wine is produced for the purpose of correcting deficiencies of these constituents, but only to the extent that such constituents would be present in normal wines of the same class or type not so treated.

Sec. 23. *Grape type designations.*—(a) A name indicative of a variety of grape may be employed as the type designation of a grape wine if the wine derives its predominant taste, aroma, and characteristics, and at least 51 per cent of its volume, from that variety of grape. If such type designation is not known to the consumer as the name of a grape variety, there shall appear in direct conjunction therewith an explanatory statement as to the significance thereof.

Sec. 24. *Generic, semi-generic, and non-generic designations of geographic significance.*—(a) A name of geographic significance which is also the designation of a class or type of wine, shall be deemed to have become generic only if so found by the Administrator.

Examples of generic names, originally having geographic significance, which are designations for a class or type of wine are: vermouth, sake.

(b) A name of geographic significance, which is also the designation of a class or type of wine, shall be deemed to have become semi-generic only if so found by the Administrator. Semi-generic designations may be used to designate wines of an origin other than that indicated by such name only if there appears in direct conjunction therewith an appropriate appellation of origin disclosing the true place of origin of the wine.

Examples of semi-generic names which are also designations for types of grape wine are: Angelica, Burgundy, Claret, Chablis, Champagne, Chianti, Malaga, Marsala, Madeira, Moselle, Port, Rhine Wine (syn. Hock), Sauterne, Haut Sauterne, Sherry, Tokay.

(c) A name of geographic significance, which has not been found by the Administrator to be generic or semi-generic, may be used only to designate wines of the origin indicated by such name, but such name shall not be deemed to be the distinctive designation of a wine unless the Administrator finds that it is known to the consumer and to the trade as the designation of a specific wine of a particular place or region, distinguishable from all other wines.

Examples of non-generic names which are not distinctive designations of specific wines are: American, California, Lake Erie Islands, Napa Valley, New York State, French, Spanish.

Examples of non-generic names which are also distinctive designations of specific grape wines are: Bordeaux Blanc, Bordeaux Rouge, Graves, Medoc, St. Julien, Chateau Yquem, Chateau Margaux, Chateau Lafite, Pommard, Chambertin, Montrachet, Rhone, Liebfraumilch, Rudesheimer, Forster Deidesheimer, Schloss Johannisberger, Lagrima, Lacryma Christi.

Sec. 25 *Appellations of origin*—(a) A wine shall be entitled to an appellation of origin if (1) at least 75 per cent of its volume is derived from fruit or other agricultural products both grown and fermented in the place or region indicated

by such appellation, (2) it has been fully manufactured and finished within such place or region, and (3) it conforms to the requirements of the laws and regulations of such place or region governing the composition, method of manufacture, and designation of wines for home consumption.

(b) Wines subjected to cellar treatment outside the place or region of origin under the provisions of section 22(c) of this article, and blends of wines of the same origin blended together outside the place or region of origin (if all the wines in the blend have a common class, type, or other designation which is employed as the designation of the blend), shall be entitled to the same appellation of origin to which they would be entitled if such cellar treatment or blending took place within the place or region of origin.

References on Wine

Amann, P. E., Chemical Sterilization in the Winery, *Fruit Products Jour.*, **13**: 177 (1931).

Allen, H. W., "The Wines of France," T. Fisher Unwin, London, 1924.

——: "The Romance of Wine," E. P. Dutton & Company, New York, 1932.

Amerine, M. A., Determination of Esters in Wines—Liquid-liquid Extraction, *Food Research*, **9** (No. 5): 392-395 (1944).

——: The Composition of California Wines at Exhibitions, *Wines & Vines*, **28** (No. 1): 21-23, 42-43, 45, (No. 2) 21-26, (No. 3) 23-25, 42-46 (1947).

—— and W. DeMattei: Color in California Wines, III. Methods of Removing Color from the Skins, *Food Research*, **5** (No. 5): 509-519 (1940).

—— and W. C. Dietrich: Glycerol in Wines, *Jour. Assoc. Offic. Agr. Chemists*, **26** (No. 3): 408-413 (1943).

—— and M. A. Joslyn: Commercial Production of Table Wines, *Bull. 639, Univ. Calif.*, July, 1940.

——, L. P. Martini, and W. DeMattei: Foaming Properties of Wine, *Ind. Eng. Chem.*, **34**: 152 (1942).

—— and A. D. Webb: Alcohol-Glycerol Ratio of California Wines, *Food Research*, **8** (No. 4): 280-285 (1943).

—— and A. J. Winkler: Maturity Studies with California Grapes I. The Balling-Acid Ratio of Wine Grapes, *Proc. Am. Soc. Hort. Sci.*, **38**: 379-387 (1940).

—— and ——: Maturity Studies with California Grapes II. The Titratable Acidity, pH, and Organic Acid Content, *Proc. Am. Soc. Hort. Sci.*, **40**: 313-324 (1942).

—— and ——: Color in California Wines IV. The Production of Pink Wines, *Food Research*, **6** (No. 1): 1-14 (1941).

—— and ——: Composition and Quality of Musts and Wines of California Grapes, *Hilgardia*, **15** (No. 6): 493-673 (1944).

Arnold, P.: The New Wine Book. The Biology of Wine Fermentation and Its Practical Importance. Western Printing Corp. Whittier California 1936.

Ash, C. S. Metals in Wineries, *Ind. Eng. Chem.* **27** 1247 1935

Association of Official Agricultural Chemists "Official and Tentative Methods of Analysis," 6th ed., Washington D. C. 1945

Babo A. F. von and E. Mach "Handbuch des Weinbaues und der Kellerwirtschaft" 4 vols. Paul Parey Berlin 1923-1927

Baines C. A. H. L. Guittard and C. S. Pederson Preservation of Grape Juice *Food Research* **3** 467 (1938)

BIGELOW, W. D.: The Composition of American Wines, *U.S Dept. Agr., Bur. of Chem., Bull* 59, 1900

BIOLETTI, F. T.: Conditioning Wine Containers, *Calif. Grape Grower*, 24: 25 (1934)

——— "Wine Making on a Small Scale," *Calif. Grape Grower*, San Francisco, 1934

BOSWELL, P : "Wine Makers Manual," Orange Judd Publishing Co., Inc., New York, 1935

BOUTARIC, A., L. FERRÉ, et M. ROY: Recherches spectrophotométriques sur la couleur des vins, *Ann Fals*, 30: 196 (1937)

BROWN, E. M, and V DEF HENRIQUES: Vinification in California Wineries, *Ind Eng. Chem.*, 27: 1235 (1935)

BURDONI, M : "Trattato di Enologia," F. Natelli Strini, Albano Laziale, Italy, 1930

BUTLER, F H : "Wine and Wine Lands of the World," Coward-McCann, Inc., New York, 1926

BYRNE, J. L, L G SAYWELL, and W. V CRUESS: The Iron Content of Grapes and Wine, *Ind. Eng. Chem (Anal Ed.)*, 9: 83 (1937).

CASH, L : The Use of Inert Gas or Mineral Oil in the Storage of Wines, *Fruit Products Jour* , 16: 4 (1936).

CHAMPLIN, F M , H. E GORESLINE, and D. K. TRESSLER: Manufacture of Champagne and Sparkling Burgundy, *Ind. Eng. Chem* , 27, 1240 (1935).

CHARLEY, V L. S : The Production of Wines (Fruit), *Fruit Products Jour.*, 16: 106, 140 (1936).

COUCHE, D D : "Modern Detection and Treatment of Wine Diseases and Defects," Published in London by the author, The Avi Publishing Co , Inc. (representative), New York

COWAN, M R : Musts, Wine and Metals, *Fruit Products Jour.*, 18: 106 (1938).

COX, H E "Chemical Analysis of Foods," Chap. VII, J. & A. Churchill, Ltd , London, 1938

CRUESS, W V · "The Principles and Practice of Wine Making," 2d ed., The Avi Publishing Co , Inc , New York, 1947

———: Preparation of Yeast Starters for Wine Making, *Fruit Products Jour.*, 13: 166 (1934)

———: The Fermentation of White Wine, *Fruit Products Jour.*, 13: 230 (1934).

———: Pasteurizing .

———: The Tourne .

———: Pasteurizatio

———: Preservation of Bottled Sauterne Wines, *Fruit Products Jour* , 15: 228 (1930).

———: Wine in the Diet, *Fruit Products Jour* , 15: 260 (1936).

———: Further Data on the Effect of SO_2 in Preventing High Volatile Acidity in Wines, *Fruit Products Jour.*, 15: 291, 324 (1936)

———: Tests on Unfortified Sweet Wines, *Wine Rev* , 4: 20 (February, 1936)

———: Checking Fermentation Temperatures, *Wine Rev*, 4: 16 (April, 1936).

———: Observations of '36 Season on Volatile Acid Formation in Muscat Fermentations, *Fruit Products Jour.*, 16: 198 (1937).

———, E M BROWN, and F C FLOSSFEDER: Sweet Wines of High Alcohol Content, *Jour Ind Eng Chem* , 12: 1277 (1916).

——— and L HOHL A Method of Improving Sweet Wines, *Fruit Products Jour* , 16: 261 (1937)

———, M. A JOSLYN, and L. G SAYWELL: "Laboratory Examination of Wines and Other Fermented Fruit Products," The Avi Publishing Co., Inc., New York, 1934

————, T. Scott, H B. Smith, and L. M. Cash: A Comparison of Various Treatments of Cement and Steel Wine-tank Surfaces, *Food Research*, 2: 385–306 (1937).

————, C. Weast, and R. Gilliland: Summary of Practical Investigations on Film Yeast, *Fruit Products Jour.*, 17: 229 (1938)

Curtis, M. J : Bacterial Diseases of Wine with Reference to *Lactobacillus* and Mannitic Organisms, *Fruit Products Jour.*, 16: 174 (1937)

Douglas, H. C., and W V Cruess: A *Lactobacillus* from California Wine: *Lactobacillus Hilgardii*, *Food Research*, 1: 113 (1936)

———— and L. S McClung. Characteristics of an Organism Causing Spoilage in Fortified Sweet Wines, *Food Research*, 2: 471–476 (1937)

Dujardin, J : "Notice sur les instruments de precision, appliqués a l'oenologie," J. Dujardin, 24 rue Pavée, Paris

Eoff, J R., H. Butler, and W Melchior: Some Unusual Alcoholic Fermentations, *Ind. Eng Chem.*, 2: 1277 (1927)

Fabre, J. H., and E Bremond. Arsenic in Grape Musts and Wines, *Ann Fals.*, 31: 146 (1938).

Goresline, H E.: The Microbiology of Wine-Making, *Fruit Products Jour.*, 15: 196 (1936)

———— and R Wellington: New York Dry Red Wines, *Wines and Vines*, 17: (No 2) 9 (1936).

———— and ————: New York Champagnes, *Wines and Vines*, 17: (No 12) 5 (1936).

Guittonneau, L., et P Pacottet "Vins de Champagne et vins Mousseux," 2d ed, J. C. Baillière et fils, Paris, 1930

Hedrick, U P . "Manual of North American Grapes," State of New York, Albany

Herstein, K M., and T C Gregory: "Chemistry and Technology of Wines and Liquors," D. Van Nostrand Company, Inc, 1935.

Hewitt, J. T : "Chemistry of Wine Making," H M Stationery Office, London, 1928

Hohl, L Further Observations on Production of Alcohol by *Saccharomyces ellipsoideus* in Syruped Fermentations, *Food Research*, 3: 453 (1938)

———— and W V Cruess. Effect of Temperature, Variety of Juice, and Method of Increasing Sugar Content on Maximum Alcohol Production by *Saccharomyces Ellipsoideus*, *Food Research*, 1: 405 (1936)

Husmann, G. C · Grape Districts and Varieties in the United States, *U S Dept Agr., Farmers' Bull* 1689

Joslyn, M A · The Possibilities and Limitations of the Artificial Aging of Wine, *Fruit Products Jour*, 13: 208 (1934)

———— The Composition of Wine and Must, *Fruit Products Jour.*, 13: 326 (1934).

———— Preliminary Observations on the Mellowing and Stabilization of Wine, *Fruit Products Jour.*, 15: 10 (1935)

———— Report on Volatile Acids in Wine, *Jour Assoc Official Agr. Chem.*, 21: 106 (1938)

———— Determination of Acetaldehyde in Wines, *Ind Eng Chem.*, 10: 364 (1938)

———— and M. A Amerine Commercial Production of Dessert Wines, *Univ. Calif., Bull* 651, September, 1941

————, and W V Cruess. Elements of Wine Making, *Calif Agr. Ext Ser., Circ* 88, November, 1934

———— and R Dunn: Acid Metabolism of Wine Yeast 1. The Relation of Volatile Acid Formation to Alcoholic Fermer   , *Jour. Am Chem. Soc*, 60: 1137 (1938)

—— and G. L. MARSH: Methods of Wine Analysis, *Jour. Assoc. Official Agr. Chem.,* May, 1935.

KAYSER, L.: The Races of Yeast and Their Significance in the Bouquet of Wine, *Chimie & industrie,* Spec. No. 619–625, May, 1924.

KROEMER, K., und G. KRUMHOLZ: "Obst- und Beerenweine," Bruno Hempel, Braunschweig, Germany, 1932.

LINCOLN, H. L.: Controlled Temperature Makes Better Wines, *Ice and Refrigeration,* **90**: 111 (April, 1936).

LOESECKE, H. W. VON, H. B. MOTTERN, and G. N. PULLEY: Wines, Brandies and Cordials from Citrus Fruits, *Ind. Eng Chem,* **28**: 1224 (1936).

MARSH, G. L.: The Peculiarities of Carbon Dioxide in Wine, *Wine Rev.,* **4**: 17 (1936).

—— and M. A. JOSLYN: Precipitation Rate of Cream of Tartar from Wine, *Ind. Eng Chem,* **27**: 1252 (1935).

MORRIS, M. M.: Volatile Acids of Wine, *Ind. Eng. Chem,* **27**: 1250 (1935).

MRAK, E. M., I. CASH, and D. C. CAUDRON: Corrosion of Metals by Musts and Wines, *Food Research,* **2**: 439–454 (1937)

——, ——, and ——: Effects of Certain Metals and Alloys on Claret- and Sauterne-Type Wines Made from Vinifera Grapes, *Food Research,* **2**: 539 (1937).

—— and J F FESSLER: Changes in Iron Content of Musts and Wines during Vinification, *Food Research,* **3**: 307 (1938).

MÜLLER-THURGAU, H., und A. OSTERWALDER: Die Bakterien im Wein und Obstwein und die dadurch verursachten Veränderungen, *Centr. Bakt. Parasitenk.,* II *Abt,* **36**: 129 (1913).

—— und ——: Weitere Beitrage zur Kenntnis der Mannitbakterien im Wein *Centr Bakt. Parasitenk,* II *Abt.,* **48**: 1 (1917).

—— und ——: Über die durch Bakterien verursachte Zersetzung von Weinsäure und Glyzerin im Wein, *Ber. Schweiz. Versuchsanstaldt, Wadensviel,* **103** (1922).

National Fruit and Cider Institute. The Production of Fruit Wines, *Fruit Products Jour.,* **16**: 140 (1937).

NELSON, E K. Flavor of Alcoholic Beverages, *Food Research,* **2**: 221 (1937).

NIEHAUS, C J. The Principal South African Wine Diseases, *Farming S. Africa,* **4**: 475 (1930)

——: Mannitic Bacteria in South African Sweet Wines, *Farming S. Africa,* **6**: 443 (1932)

OTTAVI, O.: "Enologia, Teoretico-Practico," 12th ed., Fratelli Ottavi, Casale Monferrato, Italy, 1931.

PACOTTET, P: "Vinification," J. C. Bailhère & fils, Paris, 1926

PEDERSON, C S, H E. GORESLINE, and E. A. BEAVENS: Pasteurization of New York State Wines, *Ind Eng. Chem,* **27**: 1257 (1935).

QUACCIA, L.: The Semichon Process of Fermentation, *Fruit Products Jour.,* **14**: 169 (1935).

SAYWELL, L G: Clarification of Vinegar, *Ind Eng Chem,* **26**: 379 (1934)

——: Effect of Filter Aids and Filter Materials on Wine Composition, *Ind Eng Chem,* **27**: 1245 (1935)

SCHANDERL, H: Kellerwirtschaftliche Fragen zur Schaumweinbereitung, *Wein und Rebe,* **20**: 10 (1938)

SEARLE, H E, F L LAQUE, and R H DORNOW: Metals and Wines, *Ind Eng. Chem,* **26**: 617 (1934)

SEMICHON, L. Preparation of Wine by Continuous Fermentation, *Compt. rend,* **176**: 1017 (1923)

Shand, M P.: "Book of Wines," Coward-McCann, Inc., New York, 1922

Simon, A L.: "Wine and the Wine Trade," 2d ed., Sir Isaac Pitman & Sons, London, 1931.

Stoll, H F.: California Dry White Wines, *Wine and Vines*, 17: (No 2) 14, 1936

Turbovsky, M. W., F. Filipello, W V Cruess, and P. Esau: Observations on Use of Tannin in Wine Making, *Fruit Products Jour.*, 14: 106 (1934)

U S Dept. of the Treasury, Federal Alcohol Administration, Regulations 1, Relating to the Issuance, Revocation, Suspension, and Amendment of Basic Permits, October, 1935, 1935.

U S Dept. of the Treasury, Federal Alcohol Administration, Regulations 2, Relating to Non-industrial Use of Distilled Spirits and Wine, December, 1935, 1935

U S Dept. of the Treasury, Federal Alcohol Administration, Regulations 3, Relating to Labeling and Advertising of Wine, December, 1935, 1935.

U S Dept of the Treasury, Federal Alcohol Administration under the Administration Act as in Effect on May 15, 1937, 1937.

Van Laer, M. H : "La chimie des fermentations," Masson et Cie, Paris, 1911

Ventre, J.: "Traité de vinification Pratique et rationnelle," 3 vols., Libraire A. Dubois et R. Poulain, Montpelier, France, 1930-1931.

Wagner, P. M : "American Wines and How to Make Them," Alfred A. Knopf, New York, 1933

Wiley, H W : American Wines at the Paris Exposition of 1900. Their Compositions and Character, *U S Dept Agr, Bur Chem , Bull* 72, 1903

———: "Beverages and Their Adulteration," P Blakiston's Son & Co., Philadelphia, 1919

Williams, A . Plate Heat Exchangers for the Processing of Wine *Ind Eng Jour*, 18: 294 (1930)

Winkler, A J Making Red Wines in Algeria, *Wine Rev.* (July, 1936)

———: Temperature and Varietal Interrelations in Central Valley Algeria, *Wines and Vines* (February, 1936)

——— and M A Amerine: What Climate Does, *Wine Rev.* (June, 1938)

——— and ——— Color in California Wines I Methods for Measuring Color, *Food Research*, 3: 429 (1938), II Preliminary Comparisons Factors Influencing Color, *Food Research*, 3: 439 (1938).

Winton, A L., and K B Winton: "The Structure and Composition," Vol II, John Wiley & Sons, Inc , New York, 1935

Woodman, A G : "Food Analysis," 4th ed , McGraw-Hill Book Company, Inc.

Some Patents

Buck, H G., and A W Aspen: Wine Dispensing Rack, U S Patent 2,063,610, 1936

Fowle, G W Wine Press, U S Patent 2,096,097, Oct. 19, 1937

Krebs, E T Method of Aging Wine, U S Patent 2,030,124,

Saywell, L G . Wine and Vinegar Clarification Process, U S Patent 9, 1936

Scott, A B , and G R Robertson: Wine Preserving Apparatus, U S Patent 505, Apr 7, 1936

Tressler, D K Wine Process, U S Patents 2,181,838, 2,181,839

Vingerhoets, G Producing a Wine, U S Patent 1,957,277

Williamson, W W Wine Cooling Apparatus U S Patent

THE DISTILLING INDUSTRIES

The distilling industries are those concerned with the production of distilled spirits. rum, whiskey, brandy, gin, and cordials and liqueurs.

Production Statistics.—Table 46 shows the production of distilled spirits for the fiscal year 1946, ended June 30, 1946.

TABLE 46—PRODUCTION OF DISTILLED SPIRITS, BY KINDS AND BY STATES, FISCAL YEAR 1946[1][2]

(Tax gallons)

State	Whiskey	Rum	Gin	Brandy	Other spirits	Total
Arkansas				770		770
California	379,607		313,768	33,145,469	1,520,191	35,359,035
Colorado	36,410					36,410
Connecticut	46 824		1,637		52,502	104,709
Florida				472,159		472,159
Georgia	172,971			37,322	105,164	315,457
Idaho				622,306		622,306
Illinois	15,069,588		3,025,709		19,311,498	38,306,795
Indiana	15,740,714		1,448,521		20,462,347	37,651,582
Iowa	292,586				15,471	308,037
Kentucky	63,459,394	1,141,301	199,920		20,456,577	85,257,192
Louisiana		66,535			365,691	432,226
Maine				8,534		8,534
Maryland	9,815,294		1,138,851	..	12,168,851	23,122,996
Massachusetts	1,796,681	1,133,201			3,099,236	6,029,120
Michigan					7 152,933	7,152,933
Minnesota	1,598				18 737	20,335
Missouri	1,258 688		42,048		560,821	1,861,557
New Hampshire	451,209			8,844	753,224	1,213,277
New Jersey				140,062		140,062
New York			1,081,945	51 732	4,943,095	6,076,772
Ohio	1,235 169			60,142	7,016,159	8,311,470
Oregon				223,340		223,340
Pennsylvania	35,340 916	316,541	236,159	13,918	13,214,747	49,122,281
South Dakota	132,370				250 977	383,347
Tennessee	117 591			15,810	15,881	149,282
Vermont	21,317				379,125	400,442
Virginia	1,085,653			30,267	322,079	1,437,990
Washington				213,094		213,094
Wisconsin	109 936				223,142	333,078
Total	147,464 516	2,657,580*	7,497,402	34 416,385†	113 030,754‡	305,066 637

[1] U S Treas Dept Annual Report of the Commissioner of Internal Revenue, 1946

[2] Production of whiskey, rum, and spirits by registered distilleries, and brandy and spirits by fruit distilleries

* Includes 1 551,303 tax gal removed for denaturation.

† Includes 7,005,916 tax gal of spirits-fruit

‡ Production for beverage and industrial purposes For industrial purposes 2,934,875 tax gal were produced during July, 24,961,923 tax gal during August and 2,021,823 tax gal during September, 1945

An examination of this table shows that Kentucky, Pennsylvania, Illinois, Indiana, and Maryland led the country in the production of whiskey (in the order named), while rum was produced principally in Kentucky and Massachusetts.

Illinois led the United States in the production of gin, with Indiana, Maryland, and New York following in the order named

California was outstandingly the leader in brandy manufacture, while Indiana led in the production of other spirits

RUM

"Rum" is any alcoholic distillate from the fermented juice of sugarcane, sugarcane syrup, sugarcane molasses, or other sugarcane by-products distilled at less than 190° proof (whether or not such proof is further reduced prior to bottling to not less than 80° proof) in such manner that the distillate possesses the taste, aroma, and characteristics generally attributed to rum, and includes mixtures solely of such distillates.[1]

Rum is manufactured in general in those countries which grow sugarcane or import molasses or other sugarcane products. It is made principally in the United States, Puerto Rico, Cuba, Jamaica, St Thomas and St Croix (Virgin Islands of the United States), British Virgin Islands, Demerara, Barbados, Uruguay, Martinique, Trinidad, Haiti, Santo Domingo, the Leeward Islands, Guadelupe, Mexico, Grenada, and Antigua

Composition.—Table 47 gives analyses of some rums from different sources

Acids (volatile and nonvolatile), esters, fusel oil, and aldehydes are regular constituents of rum. Ethyl acetate is the most common ester produced, while both acetaldehyde and furfural (C_4H_3O CHO) are always found, in traces at least, in rum.

Types.—Rums made in the United States, with certain exceptions, may be classified as heavy-bodied rums.[2] They contain only the congenerics (acids, esters, etc.) that are accumulated during distillation and aging in charred white oak barrels. Most of the rums are distilled at a proof less than 160° and aged for several years   They contain no coloring matter added by the distillers

Rums produced outside the United States, with exceptions, vary from very light bodied to very heavy bodied Cuba produces some of lightest bodied rums, while Jamaica manufactures the heaviest bodied rums outside the United States. Substances are frequently added to

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[1] Dept. of the U S. Treasury, Federal Alcohol Administration, Regulations 5, Mar 1, 1939

[2] VALAER, P., Foreign and Domestic Rum, *Ind Eng Chem*, 29: 988 (1937).

TABLE 47.—Analyses of Some Rums[1]
(Grams per 100 liters not calculated to proof)

Proof	pH	Total acid (as acetic)	Volatile acid (as acetic)	Estera (as ethyl acetate)	Fusel oil (as amyl alcohol)	Solids	Aldehydes	Furfural	Color in 0.5-in. cell	Source, comments
103.0	4.76	9.0	9.6	16.7	123.2	8	4.4	0.0	0.2	Pennsylvania, new
103.6	4.56	33.6	26.4	23.8	124.2	48	4.7	Trace	4.0	Pennsylvania, 0.5 year old
104.8	4.52	36.0	28.8	26.4	124.0	50	5.5	0.8	4.5	Pennsylvania, 1 year old
107.8	4.44	50.4	40.8	33.4	132.0	94	6.8	0.8	7.0	Pennsylvania, 2 years old
102.0	4.28	14.4	14.4	4.3	102.1	16	4.0	5.5	0.0	Kentucky, new
104.4	4.17	80.4	67.2	60.7	109.6	108	4.0	8.2	13.5	Kentucky, 1 year old
106.8	4.28	89.8	73.2	73.0	116.2	204	34.3	12.0	17.0	Kentucky, 2 years old
100.0	4.03	39.4	36.0	22.0	91.7	6	4.0	1.2	0.0	Massachusetts, new
100.4	4.42	84.0	74.0	29.0	91.5	86	6.4	1.2	7.5	Massachusetts, 0.5 year old
101.4	4.37	93.4	86.4	45.5	93.3	142	7.2	1.4	11.0	Massachusetts, 1.5 years old
102.4	4.30	98.4	86.4	51.0	96.8	162	7.2	1.6	13.0	Massachusetts, 2 years old
135.2	4.07	184.5	148.8	216.0	430.6	274	4.8	7.2	18.0	Old New England, approx 19 years old
87.3	5.03	13.2	12.0	11.4	49.3	675	1.6	0.1	14.3, caramel	Cuba, Ron Bacardi
92.8	3.03	4.8	2.4	44.0	59.8	205	7.0	0.8	13.5	Superior, gold
93.8	4.17	37.8	45.0	59.8	91.5	450	20.0	4.0	23.0, caramel	St. Croix cane juices. 2 years old
92.8	4.32	62.4	43.2	26.4	63.6	1,510	0.8	0.0	34.0, caramel	Jamaica, 13 years old
									34.0, caramel	Demerara

[1] VALAER, P., Foreign and Domestic Rum, Ind. Eng. Chem., 29: 948 (1937).

such rums to bring about the desired characteristics, one of these substances being caramel for artificial color (not all countries permit these additions, however).

Table 48 gives the analyses of some light-, medium-, and heavy-bodied rums.

Production of Rum. *Fermentation.*—The process for the manufacture of rum is similar in many respects to that in which industrial alcohol is produced. Methods for the propagation of pure cultures of yeast, the building up of a starter, the reaction of the main mash, the concentration of the fermentable sugar, the temperature of the fermentation, and even the principal end products are similar. Some rums (Jamaica) are made by spontaneous fermentation, however.

In the manufacture of rum, the careful selection of the raw materials, the control of the fermentation, proper distillation, and the aging of the distilled product are essential for the production of a fine rum.
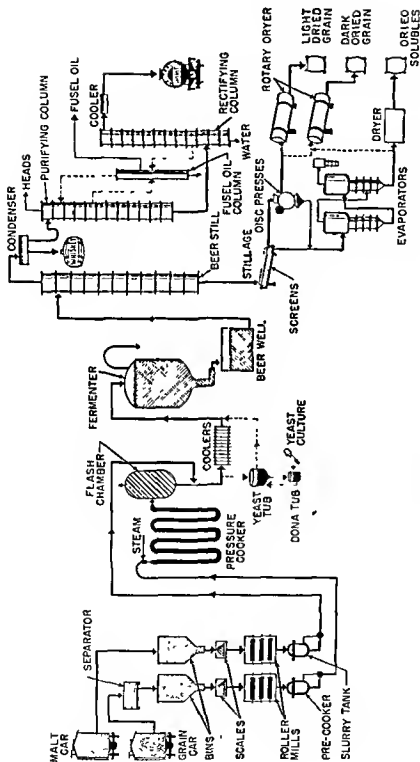
Fig 36 — Flow diagram of whiskey production   (*Courtesy of Joseph E Seagram & Sons, Louisville, Ky*)

Strains of *Saccharomyces cerevisiae* or of other yeasts, such as species of *Schizosaccharomyces*, may be used to pitch (inoculate) the mash. Great care must be exercised to maintain the selected yeast in pure culture, if the fermentation is to be successful.

As already indicated, rum is prepared from sugar-cane products   In the United States only blackstrap molasses is used in rum manufacture. This substance can be purchased at relatively low cost when procured in large quantities.   It is a product of the sugar mill after the crystallizable sugar has been removed, and usually contains 40 to 55 per cent of fermentable sugar.

TABLE 18 — ANALYSES OF SOME LIGHT-, MEDIUM-, AND HEAVY-BODIED RUMS[1]

Type of rum	Manufacture and location	Grams per 100 Liters of 100 proof								
		Proof	Total acid (as acetic)[2]	Esters (as ethyl acetate)	Alde-hyde	Fur-fural	Fusel oil (as amyl alcohol)	Color	Wood extract	
Light bodied	Bacardi (Cuba), Carta Blanca	89.6	9.1	11.7	5.4	0.1	65.0	Light	Slight	Bottled for sale in the United States
	Castillo (Cuba), Carta Oriente[3]	87.8	0.7	7.0	7.3	0.1	79.0	Dark	Slight	Bottled for sale in the United States
Medium bodied	Ron Rico (Puerto Rico), White Label[4]	82.9	28	32	19	0.6	136	Light	Present	Bottled for sale in the United States
	Bacardi (Cuba) "Extra Superior, 1873"[5]	90.1	53.	25	11.	0.8	80.	Dark	Present	Bottled for sale in the United States
Heavy bodied	Wray's J Dagger (Jamaica)[6]	99.8	34.0	56.0	18.0	4.0	228.	Dark	Present	For the United States trade. Bottled sample
	(Jamaica) for sale to Germany[7]	149	141	565.	19.0	5.4	124.	Dark	Present	High-proof. From a barreled sample

[1] Courtesy of Dr Walter C Tobie
[2] Typical of a very light-flavored Cuban rum, such as is frequently used for highballs and cocktails.
[3] Although the color is dark, the flavor of this rum is light.
[4] An early sample (about 1934) for shipment to the United States.
[5] This is one of the heaviest Cuban rums. The flavor is relatively rich and fruity.
[6] A typical Jamaica rum for sale in the United States. The flavor is high
[7] Bulk rum, said to have been prepared in Jamaica for the German trade. Note the excessively high ester value.

A concentration of molasses that contains from 12 to 14 per cent of fermentable sugars may be used in preparing the main mashes. Ammonium sulphate, and occasionally phosphates, may be added as a source of food for the yeast. Dunder, i.e., distillation slops which may be decomposed, may be used to give the rum a heavier flavor and to supply nutrient material.

Sulphuric acid is used to adjust the pH of the mash to 4.0 to 4.7, the latter pH favoring a rapid fermentation and the production of a light-flavored rum. The use of lower pH values favors slower fermentations and the production of heavy-bodied rums, such as the Jamaica type It is usually necessary to add acid to the mash, unless it has been heat sterilized, to inhibit the development of undesirable bacteria.

The fermentation tanks must be cleaned carefully after being used if a clean fermentation and light-bodied rum are desired. Steam, sulphuric acid, ammonium bifluoride, or other agents may be used to prevent the development of undesirable flora in the tank.

A fermentation temperature of about 70°F. (initial) to 90°F. (final) is satisfactory. Low temperatures favor a slow fermentation, while at high temperatures volatile constituents are lost.

Although the main fermentation is nearly complete within 2 days, the mashes are permitted to ferment for 3 to 7 days before being distilled. A 6-day or 144-hr. fermentation period is used in at least one modern distillery in this country

The fermented mash is sometimes designated as "beer"

Distillation—Distillation is an important process in the production of rum Mashes that are distilled at high proof produce light-flavored rums, which are expected to be consumed shortly. Only small changes take place in such products during storage, for the rums contain but small quantities of acids, esters, fusel oil, and other congeneric substances. As stated earlier, most rums in this country are distilled at a proof of less than 160°.

Various types of distillation equipment are used: pot stills, continuous-type stills with combined rectifying columns, beer stills with doublers, chambered stills, and other modifications

In the United States, rum distilled at a low proof is usually adjusted to 100° proof before being aged

Aging.—Aging is a process for improving the character of rums, for decreasing the undesirable flavors, and for greatly increasing the pleasant ones. Flavor, color, and aroma are developed, while the product is mellowed. During the first 6 months of the process, the main increases in esters, acids, solids, and color take place. In a freshly distilled rum, the quantity of esters usually exceeds that of the acids, but after storage

for less than 6 months the ratio changes. Indeed, the acid content is usually greater than the ester content after two years,[1] owing largely to the acidic products extracted from the wood of the barrel.

Rum is usually aged in charred white oak barrels, although some is aged in plain cooperage. The aging is permitted to proceed for a few months or for several years (in Government bonded warehouses in the United States).

Some rum is "quick-aged" by placing charred white oak chips in the rum before it is stored. Quick-aging may also be accomplished by circulating the rum over oak chips. Heat and aeration are sometimes used. Quick-aging processes are not particularly satisfactory, however.

Government Supervision.—The manufacture of rum in the United States and its possessions is carried out under Government supervision. The quantity of raw material used and rum produced are carefully measured. The Bureau of Internal Revenue is interested in taxes, and the Federal Alcohol Administration in the promulgation of regulations concerning standards for identification, such as labeling and advertising

Labeling.—According to the Federal Alcohol Administration Division,[2] a bottled rum should contain the following information on the label: the class and type, the alcoholic content, the net contents, and the presence of "artificial or excessive coloring or flavoring" A statement concerning the age of the rum may also be placed on the label, if desired

Uses.—Rum may be used in the preparation of ice cream, candies, and mincemeat; in the curing of tobacco; as a beverage; and as a medicinal.

Definitions and Regulations.—"New England rum" is rum that is produced in the United States, is distilled at less than 160° proof and is a straight rum and not a mixture of rums.[3]

Puerto Rico, Cuba, Demerara, Barbados, St. Croix, St. Thomas, Virgin Islands, Jamaica, Martinique, Trinidad, Haiti, and San Domingo rum are not distinctive types of rum. Such names are not generic but retain their geographic significance They may not be applied to rum produced in any other place than the particular region indicated in the name, and may not be used as a designation of a product as rum, unless such product is rum as defined [see page 223].

Imitation rum—(1) neutral spirits or other distilled spirits which have been added thereto or which contain synthetic or imitation rum flavoring materials, and (2) rum which has added thereto neutral spirits or other distilled spirits than rum are "imitation rum" and shall be so designated

[1] VALAER, loc. cit.

[2] U.S. Dept of the Treasury, Federal Alcohol Administration, Regulations 5, Mar. 1, 1939

[3] Ibid.

WHISKEY

According to Article II, Section 21, Class 2 of the Federal Alcohol Administration Act, Regulations 5,[1]

"Whisky" is an alcohol distillate from a fermented mash of grain distilled at less than 190° proof in such manner that the distillate possesses the taste, aroma, and characteristics generally attributed to whisky, and withdrawn from the cistern room of the distillery at not more than 110° proof and not less than 80° proof, whether or not such proof is further reduced prior to bottling to not less than 80° proof, and also includes mixtures of the foregoing distillates for which no specific standards of identity are prescribed herein .

Types.—There are several types of whiskey. Standards for the identity of American types and some others are stated later in this chapter

Composition of Whiskies.—Whiskies contain approximately 50 per cent by volume ethyl alcohol The flavor and bouquet are due mainly to the impurities or congeneric substances that they contain, however. These accumulate during the production and storage of the whiskey.

In whiskey are found acids, esters, aldehydes, furfural, fusel oil, and solids, in addition to ethyl alcohol and water. The principal acids are acetic and valeric acids,[2] but traces of propionic and other acids are found also The combination of ethyl alcohol with these acids yields ethyl acetate, ethyl valerate, and ethyl propionate, respectively, while the amyl alcohols present in the fusel oil form amyl acetate, amyl valerate, and other esters. Aldehydes include acetaldehyde and those resulting from the oxidation of small amounts of some of the higher alcohols during aging Fusel oil is composed mainly of higher alcohols.

In Table 49 are given the average analyses for 31 different samples (barrels) of whiskey, of which 14 were rye whiskey, 13 being prepared by the sweet-mash and 1 by the sour-mash method, and of which 17 were bourbon whiskey, 13 being prepared by the sour-mash and 4 by the sweet-mash method. The average data for the rye and bourbon whiskies are also given. The effect of aging on the quantities of the various components of whiskey is illustrated The results are expressed in grams per 100 liters, calculated to the original volume of the whiskey The acids are calculated as acetic acid, the esters and aldehydes as acetic, the fusel oil as amyl alcohol

Production of Whiskey.—The flavor, aroma, and characteristics of the final product depend on the nature of the raw materials and the fermentation, the method of distillation, and the aging process

[1] *Ibid*

[2] Woodman, A. G., "Food Analysis," 4th ed , McGraw-Hill Book Company, Inc , 1941.

for less than 6 months the **ratio changes**. Indeed, the acid content is usually greater than the ester **content after** two years,[1] owing largely to the acidic products extracted from the wood of the barrel.

Rum is usually aged in charred white oak barrels, although some is aged in plain cooperage. The aging is permitted to proceed for a few months or for several years (in Government bonded warehouses in the United States).

Some rum is "quick-aged" by placing charred white oak chips in the rum before it is stored. Quick-aging may also be accomplished by circulating the rum over oak chips. Heat and aeration are sometimes used Quick-aging processes are not particularly satisfactory, however.

Government Supervision.—The manufacture of rum in the United States and its possessions is carried out under Government supervision. The quantity of raw material used and rum produced are carefully measured. The Bureau of Internal Revenue is interested in taxes, and the Federal Alcohol Administration in the promulgation of regulations concerning standards for identification, such as labeling and advertising.

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[1] VALAER. *loc cit.*
[2] U.S. Dept of the Treasury, Federal Alcohol Administration. Regulations 5, Mar. 1, 1939
[3] *Ibid.*

WHISKEY

According to Article II, Section 21, Class 2 of the Federal Alcohol Administration Act, Regulations 5,[1]

"Whisky" is an alcohol distillate from a fermented mash of grain distilled at less than 190° proof in such manner that the distillate possesses the taste, aroma, and characteristics generally attributed to whisky, and withdrawn from the cistern room of the distillery at not more than 110° proof and not less than 80° proof, whether or not such proof is further reduced prior to bottling to not less than 80° proof; and also includes mixtures of the foregoing distillates for which no specific standards of identity are prescribed herein. .

Types.—There are several types of whiskey Standards for the identity of American types and some others are stated later in this chapter.

Composition of Whiskies.—Whiskies contain approximately 50 per cent by volume ethyl alcohol The flavor and bouquet are due mainly to the impurities or congeneric substances that they contain, however These accumulate during the production and storage of the whiskey.

In whiskey are found acids, esters, aldehydes, furfural, fusel oil, and solids, in addition to ethyl alcohol and water. The principal acids are acetic and valeric acids,[2] but traces of propionic and other acids are found also The combination of ethyl alcohol with these acids yields ethyl acetate, ethyl valerate, and ethyl propionate, respectively, while the amyl alcohols present in the fusel oil form amyl acetate, amyl valerate, and other esters Aldehydes include acetaldehyde and those resulting from the oxidation of small amounts of some of the higher alcohols during aging. Fusel oil is composed mainly of higher alcohols

In Table 49 are given the average analyses for 31 different samples (barrels) of whiskey, of which 14 were rye whiskey, 13 being prepared by the sweet-mash and 1 by the sour-mash method, and of which 17 were bourbon whiskey, 13 being prepared by the sour-mash and 4 by the sweet-mash method The average data for the rye and bourbon whiskies are also given. The effect of aging on the quantities of the various components of whiskey is illustrated The results are expressed in grams per 100 liters, calculated to the original volume of the whiskey. The acids are calculated as acetic acid, the esters and aldehydes as acetic, the fusel oil as amyl alcohol

Production of Whiskey.—The flavor, aroma, and characteristics of the final product depend on the nature of the raw materials and the fermentation, the method of distillation, and the aging process.

[1] *Ibid*

[2] WOODMAN, A. G., "Food Analysis," 4th ed , McGraw-Hill Book Company, Inc , 1941

1. *Raw Materials.*—Grains are the raw materials from which whiskies are produced, the usual ones being rye, corn, wheat, and barley.

In the United States, rye and bourbon are the principal types of whiskey manufactured. Rye whiskey is generally prepared from rye and rye malt or from rye and barley malt. A typical mash may contain 80 per cent rye and 20 per cent barley malt. At least 51 per cent rye is

TABLE 49.—AVERAGE ANALYSES OF SOME WHISKIES[1]
(Grams per 100 liters calculated to original volume)

Age, years	Type	Color	Solids	Acids	Esters	Alde-hydes	Furfural	Fusel oil
New	Whole	....	20 0	6.4	15.0	4.03	0.71	06.8
	Rye	....	13 6	4.7	13.7	4 91	0.97	83.2
	Bourbon	. . .	26.0	7.7	17.2	3.26	0.44	108.6
1	Whole	7.3	101 5	37 8	20.9	7.08	1.5	106 2
	Rye	8 4	114 6	41 8	35.3	8.71	1.7	106.8
	Bourbon	0.4	90.1	34 4	24.9	5.55	1 3	105 8
2	Whole	8 6	124 2	46.1	42 9	8.34	1.7	108.1
	Rye	10 6	133 6	49 8	49 3	9.02	1.0	109.7
	Bourbon	6 7	114 8	42.7	37.3	7.78	1.4	107.3
3	Whole	10 2	140 2	51.1	48.4	0 47	1.8	106.3
	Rye	11 5	150 4	54 4	54.3	0.80	2 2	104.4
	Bourbon	8 3	130 7	47 8	42.5	0 15	1.5	107 3
4	Whole	10 2	140 4	51 6	50 9	10 2	1.9	104 3
	Rye	11 6	153 1	54 2	57.2	11.2	2.2	102.0
	Bourbon	8 9	127 7	48.9	45 0	9.3	1.5	106 3
5	Whole	11 1	149 2	52.2	51 1	10 2	1 9	100.4
	Rye	12 2	158 8	54 8	57 5	11 3	2 5	100.1
	Bourbon	10 0	140 2	49 8	45.0	0.2	1 5	100 7
6	Whole	11 1	151 4	53 2	50 7	10 2	1 9	104.7
	Rye	12 3	161.0	54 8	55.5	11 3	2 4	105 9
	Bourbon	10 1	142 5	51.8	45.2	9 1	1 4	103 8
7	Whole	11 1	154 0	52.2	51.1	9 8	1 8	99.9
	Rye	12 0	161 3	51 9	56 6	10 6	2 2	98.8
	Bourbon	10 2	147 1	52 4	46.4	9.0	1.4	101.6
8	Whole	10 5	155 2	53 1	50 9	9 6	1.8	98.0
	Rye	11 1	163 8	52 6	56 7	10 6	2.2	99.0
	Bourbon	10 0	147 7	53 6	45.9	8 8	1.5	97.1

[1] CRAMPTON, C A., and L M TOLMAN. *Jour. Am. Chem. Soc.* **30**: 98 (1908).

required by law. Bourbon, on the other hand, is prepared from corn (maize or Indian), barley malt or wheat malt, and usually another grain. A typical mash may contain, for example, 70 per cent corn, 15 per cent rye and 15 per cent malt, or 65 per cent corn, 23 per cent rye and 12 per cent malt. The mash must always contain at least 51 per cent corn (see the standards of identity for whiskey given in this section).

The preparation of a mash for whiskey is similar in fundamental details to the preparation of a mash in the brewing industry, except that the solids are not removed (Chap. VI). The enzymes of the malt convert the starches of the cooked grains to dextrins and sugars. At the same time, the protein molecules are degraded sufficiently to furnish the yeast with a satisfactory source of nitrogen.

2. *Fermentation.*—The resultant mash is fermented by a strain of distiller's yeast, *Saccharomyces cerevisiae*, by either the sweet- or sour-mash method. In the former method, which is commonly used, the mash is inoculated directly with the yeast. Less time is required for this fermentation, generally, and a higher yield of alcohol is obtained than from fermented sour mashes. In the latter method the spent slops and barm (yeast) from tanks previously set and fermented are mixed with the mash before fermentation.

Bacteria play a part in the production of esters in whiskey manufacture, the temperatures attained during fermentation favoring their growth. Not infrequently the distiller may grow his selected culture of yeast in a medium containing lactic acid bacteria, the lactic acid favoring the growth of yeast, inhibiting certain undesirable types of microorganisms, and contributing to the aroma, flavor, and characteristics of the whiskey.

3. *Distillation.*—Both the type of still and the method of distillation are important, for they contribute to the quality of the product. The continuous still, with doubler, and the three-chambered still, with doubler, are two commonly used stills.

4. *Aging.*—During the process of aging, definite changes take place in certain of the congeneric substances of whiskey as the result of storing it in oak containers. These changes have been investigated in great detail by Crampton and Tolman (1908) and by Valaer and Frazier (1936).

"Age" for "American type whiskeys, other than corn whiskey, straight corn whiskey, blended corn whiskey, and blends of straight corn whiskey, produced on or after July 1, 1936, means the period during which the whiskey has been kept in charred new oak containers "[1]

Whiskey is stored in heated or unheated warehouses for aging, heat causing greater extraction of substances from the wood of the container.

CHANGES DURING AGING.—During aging in white oak barrels, changes take place in the solids, esters, acids, fusel oil, aldehydes, furfural, and color, the largest increase in solids, esters, acids, and color occurring during the first half year of storage.[1]

The solids of whiskey, known also as extract, are derived from the wood of the container. During the first 6 months of storage, Valaer and Frazier found an average increase of about 70 g of solids per 100 liters. The quantity of extract was progressively smaller during each succeeding 6-month period, being apparently 4 g. per 100 liters during the period between the forty-second and forty-eighth months of storage.

Since there is very little change in the ester content of a whiskey during quick-aging, the determination of esters may be used as a dependable index of the age of the whiskey.

The acids of whiskey are both volatile and fixed acids. The larger part of the increase in total acids during storage is due to volatile acids. Some fixed acids are extracted from the wood of the container.

During storage in charred oak barrels, there is an actual loss in the quantity of fusel oil, according to Valaer and Frazier. The fusel-oil determination gives information concerning the method of distillation and is, therefore, considered to be an important determination.

There is an actual loss of aldehydes during storage.

Some furfural is extracted from charred oak barrels during the first 6 months of storage, but very little appears later. Uncharred barrels do not contribute furfural.

QUICK-AGING.—The process of quick-aging of whiskey is used extensively, especially for whiskies that are to be marketed shortly after manufacture. Quick-aging produces but little change in the ester content and no effect on the fusel oil. It increases the aldehydes only slightly. Solids, acids (mainly the nonvolatile ones), furfural, and color are increased. Color depth is readily varied by means of this process. The following table illustrates the effect of quick-aging on a whiskey

Heat, charred or uncharred wood chips, and charred barrels are used in some of the quick-aging processes.

CHANGES IN GLASS.—Although extensive changes do not usually occur in whiskies stored in glass containers, evidence indicates that there may be a slight decrease in acids in the case of some whiskies, especially those which are not new. Valaer and Frazier suggest that this reduction in acidity may be due to the alkali dissolved from the glass, to the establish-

[1] VALAER, P., and W H FRAZIER, *Ind Eng Chem*, 28: 92 (1936).

TABLE 50.—DIFFERENCE IN SAME WHISKY WITH AND WITHOUT QUICK-AGING[1]

	Grams per 100 liters calculated to proof	
	Quick-aged	Not quick-aged
Total acids . .	11 9	5 9
Esters. .	14 3	15 6
Fusel oil. .	124 3	127 6
Solids. .	33 6	11 2
Color	3 5	0 0
Furfural	1 0	0 0

[1] VALAER, P., and W. H. FRAZIER, *Ind Eng Chem* **28**, 92 (1936).

ment of a change in equilibrium, or to an oxidation change Esters tend to increase as does the color, while furfural is reduced in quantity.

Federal Control.—The manufacture of whiskey is carried out under Government supervision from the weighing of the grain to the sale of the whiskey. The Bureau of Internal Revenue and the Federal Alcohol Administration Division are much interested in the control of the whiskey production. For details in connection with importing, bottling, labeling, and advertising, the reader is referred to the publications of the latter division, some of which are listed in the references given at the end of this section

Standards of Identity.—Standards of identity for different types of whiskies, under the provisions of the Federal Alcohol Administration Act, are as follows ("American type" whiskies being specified in subsection (*a*) through (*j*)).

(*b*) "Rye whiskey," "bourbon whiskey," "wheat whiskey," "malt whiskey," or "rye malt whiskey" is whiskey which has been distilled at not exceeding 160° proof from a fermented mash of not less than 51% rye grain, corn grain, wheat grain, malted barley grain or malted rye grain, respectively; and, if produced on or after March 1, 1938, stored in charred new oak containers, and also includes mixtures of such whiskeys where the mixture consists exclusively of whiskeys of the same type. "Corn whiskey" is whiskey which has been distilled at not exceeding 160° proof from a fermented mash of not less than 80% corn grain, stored in uncharred oak containers or reused charred oak containers, and not subjected, in the process of distillation or otherwise, to treatment with charred wood, and also includes mixtures of such whiskey

(*b*) "Straight whiskey" is an alcoholic distillate from a fermented mash of grain distilled at not exceeding 160° proof and withdrawn from the cistern room of the distillery at not more than 110° and not less than 80° proof, whether or not such proof is further reduced prior to bottling to not less than 80° proof, and is—

(1) Aged for not less than **twelve calendar** months if bottled on or after July 1, 1936, and before July 1, 1937; or

(2) Aged for not less than **eighteen calendar** months if bottled on or after July 1, 1937, and before July 1, 1938; or

(3) Aged for not less than **twenty-four calendar** months if bottled on or after July 1, 1938.

The term "straight whiskey" also includes mixtures of straight whiskey which, by reason of being homogeneous, are not subject to the rectification tax under the Internal Revenue Laws.

(c) "Straight rye whiskey" is straight whiskey distilled from a fermented mash of grain of which not less than 51% is rye grain.

(d) (1) "Straight bourbon whiskey" is straight whiskey distilled¹ from a fermented mash of grain of which not less than 51% is corn grain.

(3) "Straight corn whiskey" is straight whiskey distilled from a fermented mash of grain of which not less than 80% is corn grain, aged for the required period in uncharred oak containers or reused charred oak containers, and not subjected, in the process of distillation or otherwise, to treatment with charred wood.

(e) "Straight wheat whiskey" is straight whiskey distilled from a fermented mash of grain of which not less than 51% is wheat grain.

(f) "Straight malt whiskey" and "straight rye malt whiskey" are straight whiskey distilled from a fermented mash of grain of which not less than 51% of the grain is malted barley or malted rye, respectively.

(g) "Blended whiskey" (whiskey—a blend) is a mixture which contains at least 20% by volume of 100° proof straight whiskey and, separately or in combination, whiskey or neutral spirits, if such mixture at the time of bottling is not less than 80° proof.

(h) "Blended rye whiskey" (rye whiskey—a blend), "blended bourbon whiskey" (bourbon whiskey—a blend), "blended corn whiskey" (corn whiskey—a blend), "blended wheat whiskey" (wheat whiskey—a blend), "blended malt whiskey" (malt whiskey—a blend) or "blended rye malt whiskey" (rye malt whiskey—a blend) is blended whiskey which contains not less than 51% by volume of straight rye whiskey, straight bourbon whiskey, straight corn whiskey, straight wheat whiskey, straight malt whiskey, or straight rye malt whiskey, respectively.

(i) "A blend of straight whiskeys" (blended straight whiskeys), "a blend of straight rye whiskeys" (blended straight rye whiskeys), "a blend of straight bourbon whiskeys" (blended straight bourbon whiskeys), "a blend of straight corn whiskeys" (blended straight corn whiskeys), "a blend of straight wheat whiskeys" (blended straight wheat whiskeys), "a blend of straight malt whiskeys" (blended straight malt whiskeys), and "a blend of straight rye malt whiskeys" (blended straight rye malt whiskeys) are mixtures of only straight whiskeys, straight rye whiskeys, straight bourbon whiskeys, straight corn whis-

¹ U S Dept. of the Treasury, Federal Alcohol Administration, Amendment 5 to Regulations 5

keys, straight wheat whiskeys, straight malt whiskeys, or straight rye malt whiskeys, respectively.

(j) "Spirit whiskey" is a mixture (1) of neutral spirits and not less than 5% by volume of whiskey, or (2) of neutral spirits and less than 20% by volume of straight whiskey, but not less than 5% by volume of straight whiskey, or of straight whiskey and whiskey, if the resulting product at the time of bottling be not less than 80° proof.

(k) "Scotch whiskey" is a distinctive product of Scotland, manufactured in Scotland in compliance with the laws of Britain regulating the manufacture of Scotch whiskey for consumption in Great Britain, and containing no distilled spirits less than three years old: *Provided,* That if in fact such product as so manufactured is a mixture of distilled spirits, such mixture is "blended Scotch whiskey" (Scotch whiskey—a blend). "Scotch whiskey" shall not be designated as "straight."

(l) "Irish whiskey" is a distinctive product of Ireland, manufactured either in the Irish Free State or in Northern Ireland, in compliance with the laws of those respective territories regulating the manufacture of Irish whiskey for consumption in such territories, and containing no distilled spirits less than three years old: *Provided,* That if in fact such product as so manufactured is a mixture of distilled spirits, such whiskey is "blended Irish whiskey" (Irish whiskey—a blend). "Irish whiskey" shall not be designated as "straight."

(m) "Canadian whiskey" is a distinctive product of Canada, manufactured in Canada in compliance with the laws of the Dominion of Canada regulating the manufacture of whiskey for consumption in Canada, and containing no distilled spirits less than two years old *Provided,* That if in fact such product as so manufactured is a mixture of distilled spirits, such whiskey is "blended Canadian whiskey" (Canadian whiskey—a blend). "Canadian whiskey" shall not be designated as "straight."

(n) "Blended Scotch type whiskey" (Scotch type whiskey—a blend) is a mixture made outside Great Britain and composed of—

(1) Not less than 20% by volume of 100° proof malt whiskey or whiskeys distilled in pot stills at not more than 160° proof, from a fermented mash of malted barley dried over peat fire, whether or not such proof is subsequently reduced prior to bottling to not less than 80° proof, and

(2) Not more than 80% by volume of neutral spirits, or whiskey distilled at more than 180° proof, whether or not such proof is subsequently reduced prior to bottling to not less than 80° proof.

(o) "Blended Irish type whiskey" (Irish type whiskey—a blend) is a product made outside Great Britain or the Irish Free State and composed of—

(1) A mixture of distilled spirits distilled in pot stills at not more than 171° proof, from a fermented mash of small cereal grains of which not less than 50% is dried malted barley, and unmalted barley, wheat, oats, or rye grains, whether or not such proof is subsequently reduced prior to bottling to not less than 80° proof, or

(2) A mixture consisting of not less than 20% by volume of 100° proof malt whiskey or whiskeys distilled in pot stills at approximately 171° proof, from a

fermented mash of dried malted barley, whether or not such proof is subsequently reduced prior to bottling to not less than 80° proof; and

(3) Not more than 80% by volume of neutral spirits, or whiskey distilled at more than 180° proof, whether or not such proof is subsequently reduced prior to bottling to not less than 80° proof.

BRANDIES

The following standards of identity for brandies are quoted from Regulations 5 (Article II, Sec. 21, Class 4) of the Federal Alcohol Administration.[1]

(a) "Brandy" is a distillate, or a mixture of distillates, obtained solely from the fermented juice or mash of fruit (1) distilled at less than 190° proof in such manner that the distillate possesses the taste, aroma, and characteristics generally attributed to brandy; and (2) bottled at not less than 80° proof; and shall also include such distillates, aged for a period of not less than fifty years, and bottled at not less than 72° proof, in cases where the reduction in proof below 80° is due solely to losses resulting from natural causes during the period of aging.

(b) "Brandy," without appropriate qualifying words, or "grape brandy," is the distillate obtained from grape wine or wines under the conditions set forth in subsection (a) of this class, and includes brandies solely of such distillates.

(c) "Apple brandy" (apple jack), "peach brandy," "cherry brandy," "apricot brandy," "orange brandy," "raisin brandy," and other fruit brandies are distillates obtained from the fermented juice or mash of the respective fresh or dried or otherwise treated fruits under the conditions set forth in subsection (a) of this class, and include mixtures composed wholly of one kind of such distillates. The designation shall contain the name of the fruit used, and, if other than whole fresh fruit is used, the word "dried" or such other term as may be appropriate. Brandy derived from raisins shall be designated as "raisin brandy."

(d) "Cognac" or "Cognac brandy" is grape brandy distilled in the Cognac Region of France, which is entitled to be designated as "Cognac" by the laws and regulations of the French Government; and includes mixtures of such brandy.

Brandies usually contain 40 to 50 per cent ethyl alcohol by volume, but may sometimes contain as little as 36 per cent alcohol by volume [paragraph (a) above]

A large amount of the wine manufactured in California (more than one-half, according to Goresline),[2] is distilled to produce brandy. Approximately 75 per cent of such brandy is used to fortify wines.

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[1] U S Dept. of the Treasury, Federal Alcohol Administration, Regulations 5, Mar. 1, 1939

[2] GORESLINE, H E., Notes on Wine Manufacture in the United States, U S Dept. Agr (mimeographed sheets), 1936.

GINS

(a) "Distilled gin" is a distillate obtained by original distillation from mash, or by the redistillation of distilled spirits, over or with juniper berries and other aromatics customarily used in the production of gin, and deriving its main characteristic flavor from juniper berries and reduced at time of bottling to not less than 80° proof, and includes mixtures solely of such distillates.

(b) "Compound gin" is the product obtained by mixing neutral spirits with distilled gin or gin essence or other flavoring materials customarily used in the production of gin, and deriving its main characteristic flavor from juniper berries and reduced at time of bottling to not less than 80° proof, and includes mixtures of such products.

(c) "Dry gin," "London dry gin," "Hollands gin," "Geneva gin," "Old Tom gin," "Tom gin," and "buchu gin" are the types of gin known under such designations, and shall be further designated as "distilled" or "compound," as the case may be.[1]

As noted above, gins contain at least 40 per cent ethyl alcohol by volume

The basic flavoring of the best gins are produced by juniper berries, coriander seed, fennel seed, sweet orange, and cassia bark [2]

CORDIALS AND LIQUEURS

(a) Cordials and liqueurs are products obtained by mixing or redistilling neutral spirits, brandy, gin, or other distilled spirits with or over fruits, flowers, plants, or pure juices therefrom, or other natural flavoring materials, or with extracts derived from infusions, percolations, or maceration of such materials, and to which sugar or dextrose or both have been added in an amount not less than 2½% by weight of the finished product Synthetic or imitation flavoring materials shall not be included.

(b) "Sloe gin" is a cordial or liqueur with the main characteristic flavoring derived from sloe berries

(c) Cordials and liqueurs shall not be designated as "distilled" or "compound "

(d) Dry cordials and dry liqueurs—The designation of a cordial or liqueur may include the word "dry" if the added sugar and dextrose are less than 10% by weight of the finished product [3]

[1] U S Dept of the Treasury, Federal Alcohol Administration, Regulations 5, Art II, Sec 21, Class 3, 1939

[2] Xugry, A L, Modern Gin Production, *Am Wine Liquor Jour.*, p 42, April, 1934

[3] U S Dept of the Treasury, Federal Alcohol Administration, Regulations 5, Art II, Sec 21, Class 6, 1939

References on the Distilling Industries

Rum

Arroyo, R : Studies on Rum, *Research Bull. No 5, Univ. Puerto Rico, Agr. Expt. Sta,* Rio Piedras, P. R , December, 1945.

Tobie, W C.: Improved Procedures in Determination of Aldehydes in Distilled Alcoholic Liquors with Schiff's Reagent, *Food Research,* **3**: 499 (1938).

———: Personal communication, 1939

U S. Dept. of the Treasury, Federal Alcohol Administration, Regulations 5, Mar. 1, 1939

U S Dept. of the Treasury, Federal Alcohol Administration (Note to bottlers and importers of distilled spirits), Feb 28, 1936.

Valaer, P . Foreign and Domestic Rum, *Ind. Eng. Chem.,* **29**: 988 (1937).

Whisky

Adams, A B . The Distillation of Whiskey, *Jour. Ind. Eng. Chem.,* **2**: 31 (1910).

Anon A Modern Distillery, *Chem. & Met. Eng ,* 126-II to 129-II, November, 1942.

Anon.: "Plant Operations," The Calvert Distilling Co., Louisville, Ky., 1946.

Association of Official Agricultural Chemists. "Official and Tentative Methods of Analysis," 6th ed , Washington, D C., 1945

Boruff, C S "Microbiology in a Modern Whiskey Distillery," p 333, Abstracts of Communications, Third International Congress for Microbiology, September, 1939

Brown, T F Whiskey Distilling, *Am. Wine Liquor Jour.* (September, 1937).

——— and H H Schopmeyer. The Production of Bourbon, *Am. Wine Liquor Jour.,* p 54 (December, 1935)

Foth, G "Handbuch der Spiritusfabrikation," Paul Parey, Berlin, 1929.

Gallagher, M , P Kolachov, and H F Wilkie: Whiskey Losses during Aging, *Ind. Eng Chem ,* **34**: 992-995 (1942)

Hirsch, I . Manufacture of Whiskey, Brandy and Cordials, 2d ed , Sherman Engineering Co , Newark, N J

Kolachov, P . The Distilling Industry in War and Peace, *Trans. Am Assoc. Cereal Chemists,* **2** (No 3) 14-23 (1944)

Owen, W L. The Amylo Process of Malting in Whiskey Distilleries, *Am. Wine Liquor Jour.* (June, 1936)

Schicktanz, S T , and A D. Etienne: Acid Content of Whiskey Variation with Age and Dilution, *Ind Eng Chem ,* **29**: 157-159 (1937)

Schmitt, R O , P J Kolachov, and H F Wilkie: Processing Equipment for Whiskey Filtration, *Ind Eng Chem ,* **34**: 1135-1138 (1942)

Unger, E D Recent Advances in Alcohol Distillation Methods, *Chem Inds ,* 778-780, May, 1946

U S Dept. of the Treasury, Federal Alcohol Administration, Regulations 5, Relating to Labeling and Advertising of Distilled Spirits, as amended to Mar. 1, 1939, 1939

U S. Dept. of the Treasury, Federal Alcohol Administration, Regulations 5, Amendment 5, Feb 28, 1938. Amendment 6, June 8, 1938

U S Dept. of the Treasury, Federal Alcohol Administration, Notice to Bottlers and Importers, Feb. 28, 1936

U S Dept. of the Treasury, Federal Alcohol Administration, A Digest of Interpretations of Regulations 5 Relating to Labeling and Advertising of Distilled Spirits, January, 1937

VALAER, P , and W H FRAZIER; Changes in Whiskey Stored for Four Years, *Ind. Eng. Chem* , **28**: 92 (1936)

WILLKIE, H. F : Distillery Processes, *Chem. & Met Eng* , May, 1945.

WOODMAN, A. G "Food Analysis," 4th ed , McGraw-Hill Book Company, Inc , New York, 1941

GENERAL

BARRON, H.: "Distillation of Alcohol," Joseph E Seagram & Sons Co , Inc , Louisville, Ky , 1944

BEAVENS, E A , H E GORESLINE, and E K NELSON Silver in the Artificial Aging of Brandies, *Ind Eng Chem* , **29**: 623 (1937).

GORESLINE, H E : "List of Selected References on Distilled Liquors and Alcohol," U.S Dept Agr , Bur Chem and Soils, Food Research Division, July, 1936

GUYMON, J. F , N E TOLBERT, and M A AMERINE Studies with Brandy 1 pH, *Food Research*, **8** (No 3): 224-230 (1943)

HERSTEIN, K M , and T. C GREGORY: "Wines and Liquors," D Van Nostrand Company, Inc , New York, 1935

JACOBSEN, E "Handbuch fur die getranke-industrie, getranke-fabriken und den getranke-gross-betrieb," Paul Parey, Berlin, 1925

JOSLYN, M A , and M A AMERINE Commercial Production of Brandies, *Bull 652, Univ Calif., Agr Expt Sta.*, Berkeley, September, 1941

MARILLER, C : "Distillation et rectification des liquides industriels," Dunod, Paris, 1925

TOBIE, W C · 3/10 of a degree for accurate proofing, *Am Wine Liquor Jour* , p 17 (April, 1939)

TOLBERT, N E , M A AMERINE, and J F GUYMON Studies with Brandy II. Tannin, *Food Research*, **8** (No 3)· 231-236 (1943)

Some Patents

FARRIER, L H , and R H HARLIN. Apparatus for Purifying and Aging Liquor, U S Patent 2,108,661, Feb 15, 1938

KRAUSE, G A Process for Artificially Aging Alcoholic Liquids and Perfumes, U.S. Patent 2,109,151, Feb 22, 1938

LIEBMANN, A. J · Method of Making Gin, U S Patent 2,152,318, Mar 28, 1939

NAGLE, C D · Aging Device for the Contents of Barrels, Casks, or the Like, U S Patent 2,135,622, Nov 8, 1938

REIMAN, C K Aging Distilled Alcoholic Spirits, U S Patent 2,132,435, Oct 11, 1938.

SHAPIRO, A · Process of Making Liquors, U S Patent 2,160,036, May 30, 1939

WILKEN, C J . Method of Producing Yeast Mash, U.S. Patent 2,132,091, Oct 4, 1938.

media containing lactic acid, while contamination, especially with butyric acid organisms, is reduced. The presence of lactic acid in the yeast cake inhibits the development of bacteria.

Aeration.—The proper aeration of the mash is of great importance in the manufacture of bakers' yeast. It is accomplished by passing air bubbles of suitable size through the entire medium.

The surface area of a given volume of bubbles of air in a liquid increases as the sizes of the bubbles become smaller. There is thus a larger contacting surface with small-sized bubbles than with large ones and a longer period of contact with the medium or wort. A smaller quantity of air is required when the bubbles used are of small size, but it costs more to produce very fine bubbles. Thus, the yeast manufacturer must select a desirable course, in which efficiency of aeration is high but not too costly. The bubbles used commercially vary from 0.0001 to 1 in. in diameter, according to de Becze and Liebman,[1] who have thoroughly discussed the subject of aeration.

Air is introduced near the bottom of the fermentation tank and the aeration network should be of such nature that there is uniform distribution of air over the entire cross section of the tank.

The air requirements for a wide and shallow fermentor tank will be much different from those of a narrow, tall tank, for the longer a bubble of air is in contact with the medium, the more effective it is. Based on the yeast produced, a considerably larger amount of air is required for a small fermentor than for a large one.

de Becze and Liebman reported that the correct evaluation of air requirements must be based on information available concerning the height of the wort at any time (obtained by reference to a chart or graph); the concentration of yeast at any time (by graph); the yield of yeast, per unit of raw material (by graph); and the ratio of the amount of new yeast to seed yeast.

Air requirements are generally reported in terms of cubic feet per (1) gallon of wort, (2) square foot of wort or fermentor bottom surface, (3) pound of yeast in 100 gal. of wort, (4) pound of yeast produced, (5) ton of total molasses, or (6) bushel of grain mashed; or milliliters, liters, or cubic meters per unit volume of medium, etc.

Aerators are of many types. For example, they may be plates, tubes, candles, spargers, or false bottoms. They may be of the fixed or movable type. They may be constructed of noncorrosive metal, ceramic substances, hard or soft rubber, porous carbon, sintered glass, canvas, or other substances. For a review of this subject the reader is referred to the article by de Becze and Liebman.

[1] DE BECZE, G., and A. J. LIEBMAN, *Ind. Eng. Chem.*, **36**: 882 (1944).

Function of Oxygen.—The exact function of oxygen in the growth of yeast is not known. Very likely its action may be due to several factors. inhibition of fermentation and increase of respiration, agitation of the medium, removal of toxic end products, and stimulation of vegetative growth.

pH.—The pH is generally adjusted to 4 4 to 4 6 However, slightly lower or slightly higher values may be used under certain conditions

The pH is maintained by one or more methods. One way is to check the pH every 30 min. or so and adjust by the addition of ammonia or sulphuric acid as indicated. Another way is to control automatically. Much may be done to control pH simply by using a suitable proportion of nutrients in the strong wort

Temperature.—The temperature is generally started at 25 or 26°C. It is usually held fairly close to this level during the early part of the fermentation and gradually permitted to rise to 30°C. towards the end of the fermentation. Cooling and heating coils are an essential part of the fermentation-tank equipment.

Preserving the Yeast.—The use of a low temperature is necessary for the storage of compressed yeast Molds and bacteria cause the cake to deteriorate rapidly at room temperature and autolysis is likely to take place.

Various methods for improving the keeping qualities of yeasts have been proposed. For example, it has been suggested[1] that washing the yeast for 1 hr. with a solution of ethyl, propyl, isopropyl, butyl, or amyl alcohol before pressing improves the keeping quality The use of colloids—pectin, agar, gelatin, gum tragacanth, dextrin, algae extract— to remove the water from yeast, followed by drying to a definite moisture content at a temperature that will not injure the yeast, has also been proposed.

Molasses-Ammonia Process.—In the molasses-ammonia process, molasses (cane and sugar beet) is used as the source of carbon and mineral elements for the growth of the yeast, while the nitrogen is supplied by ammonia, or ammonium sulphate.

The molasses mash is carefully prepared. If the molasses is deficient in phosphorus, ammonium or calcium phosphate is added Phophorus is important in the synthesis of nucleoproteins by the yeast and in buffering the medium. It stimulates growth also.

In order to produce a clear solution, the molasses-wort is filtered.

The pH of the clear, highly diluted mash is adjusted The wort is then inoculated with a starter of yeast, which has been carefully prepared from a single cell of the selected yeast.

[1] British Patent 406,398, 1934

During operation, several factors must be carefully controlled. The most important of these are the aeration, the pH, the concentration of the available sugar, and the temperature.

The availability of a large supply of air stimulates the production of yeast cells. All the air used must be sterilized before passage through the mash

The pH is maintained within a definite range, that most favorable for yeast growth (about 4.4), during the entire growth period by the addition of sulphuric acid or ammonium hydroxide. As the ammonia is used up, the mash tends to become more acid in reaction. The addition of ammonium hydroxide not only neutralizes some of the acid but also supplies more ammonium salts for the nitrogenous needs of the growing yeast.

During the process, measured portions of the molasses wort are added at specified time intervals in such quantities that the yeast produces but very little alcohol from the sugar.

It is essential to control the temperature of the mash during the growth of the yeast This may be done by the use of cooling coils set on the inside of the covered vats, which usually have capacities for several thousand gallons. The optimum temperature for the growth of the yeast strain being used is maintained.

At the end of the growing process, yeast is separated from the wort by the use of centrifugals (or by filtration). The yeast thus obtained is washed repeatedly, until the wash water appears to be quite clear. It is then pressed.

A small amount of flour (potato, tapioca, or cereal) may or may not be added to the yeast The flour absorbs some of the moisture from the yeast, increases the friability, and aids in molding the yeast into small cakes. Much yeast for the baking industries is packaged without the incorporation of starch.

A Procedure Employed in Germany.—Bakers' yeast was produced in one German plant by the following procedure[1]: Beet molasses is diluted to 30°Bé and adjusted to pH 4.5. It is then sterilized by heating at 105°C. for 1 hr. The solution is allowed to stand for 4 to 6 hr. during which time suspended solids settle out and the temperature drops to 90°C. It is filtered. Nutrients, in the form of 110 kg. of ammonium sulphate and 250 kg. of superphosphate for each 7,000 kg. of yeast produced, are added. This mixture is held in storage tanks and later fed at a slow predetermined rate to the fermentation tank.

The inoculum is prepared as follows: A strain of *S. cerevisiae*, especially suitable for the type of molasses being used, is grown on a slant. This

[1] BALLS, A K , *Fiat Final Report No 277*, Oct 3, 1945

culture is used to inoculate a 1-liter mash The size of the inoculum is progressively increased by successive transfer through 50-, 200-, 1,000-, and 15,000-liter mashes Finally a 90,000-liter inoculum is prepared which is used to inoculate six large fermentors of the type described below.

Fermentation (propagation) tanks are constructed of steel and are of 200,000-liter capacity. To start a fermentation, 20,000 liters of water are run into the tank. Sterilized, nutrient molasses is added to the water until the sugar content reaches 1.5 per cent The inoculum is then added and aeration commenced The feed of the sterile 30°Bé molasses is then adjusted so that the sugar concentration is kept at 1 5 per cent. The temperature is maintained at 25 to 30°C. The fermentation is over in 10 to 11 hr.

The aerating system consists of perforated copper tubes located at the bottom of the fermentor For each kilogram of yeast produced, 16 cu.m. of air are supplied. A supply of 4,000 cu.m. of air per hr. is necessary for 7,000 kg. of molasses. Crude lanolin is used as an antifoam agent.

The yeast is separated from the spent wort by means of Westphalia centrifuges. The cream so obtained is washed twice, cooled to 8 to 9°C., and pressed in a plate-and-frame press. The resultant cake contains 31 per cent solids. It is diluted with water to 25 per cent solids before being packaged

Yeast from Sulphite Liquor by the Heijkenskjöld Method.[1]—Sulphite liquor, mixed with a small quantity of molasses, serves as the source of raw material for the manufacture of yeast by the Heijkenskjöld method.

The first step in this process consists of adjusting the hot sulphite liquor to a pH of 6.0 to 6 5 with lime, powdered limestone, and sodium carbonate, while the liquor is being aerated in tanks After the neutralization, the liquor is permitted to stand, in order that the precipitates may settle out The clear supernatant liquor is decanted from the tanks and cooled to 28 to 30°C. by passage through coolers and is then ready for use in the manufacture of yeast.

The initial mash contains molasses mixed with sulphite liquor to yield a sugar concentration of 3 to 5 per cent. Malt sprouts may be added to this mash, if desired, to supply organic nitrogen and other nutrient substances. The mash is now inoculated with the seed yeast, a strain of *Saccharomyces cerevisiae* ("Rasse 12" of the Garungsinstitut of Berlin is used in Finland and at the Best plant in Liverpool, Nova Scotia[2]).

Sulphite liquor is fed into the mash after the yeast has commenced to develop new cells Ammonium sulphate and phosphates are added during the course of the process to furnish nutrient material and to main-

[1] LWESON, E W , *Chem Industries*, **38**: 573 (1936)
[2] *Ibid*

tain the desired reaction. Large quantities of filtered air are supplied, the air removing the carbon dioxide and other gases formed besides carrying out other functions. After the propagation is complete, the yeast is washed repeatedly. The final product is light in color, of high purity, and neutral in taste.[1]

The sulphite liquor used in this process contains approximately 25 kg. of sugar per 1,000 kg. of liquor, and of this amount about 16 kg. are fermentable. The sugar concentration is thus very low and would not favor the production of alcohol in large amounts.

An average yield of 160 per cent is obtained on the basis of the fermentable sugar, yields varying from 40 to 55 lb. for each ton of waste sulphite liquor.

TABLE 51 —COMPOSITIONS OF SOME MEDIA FOR GROWTH OF YEAST[1]

GRAIN-WORT MEDIUM

Corn.	2	kg.
Malt . .	1.5	kg.
Sprouts .	1 5	kg.
Concentrated hydrochloric acid	10	cc.
Tap water, to make .	30	liters

MOLASSES-SALTS MEDIUM

Beet molasses (crude)	150	g.
Calcium phosphate	1	g.
Ammonium monohydrogen phosphate	1	g.
Urea	2	g
Concentrated sulphuric acid	2	cc.
Tap water, to make . .	1	liter

GLUCOSE-SALTS MEDIUM

Commercial glucose .	70	g.
Sodium chloride	2	g.
Magnesium sulphate	2	g.
Calcium chloride. .	0 4	g.
Potassium pyrophosphate	2	g.
Ferric pyrophosphate	0 1	g.
Ammonium chloride	0 15	g.
Copper sulphate	Trace	
Urea (commercial)	2 4	g.
Tap water, to make	1	liter

[1] PAYCER, P L., W. H. PETERSON, and C. A. ELVEHJEM, *Ind. Eng. Chem.*, **29** : 536 (1937).

Some Media for Yeast Propagation and Their Preparation.—Table 51 presents the compositions of some media for the growth of yeast. They are prepared as follows:

1. *Grain-wort Medium.*—The finely ground corn is autoclaved at a pressure of 15 lb. per sq. in. for 0 5 hr. in the presence of about 8 liters of water At the same time the malt and sprouts are mixed in 20 liters of water, 5 cc. of concentrated HCl is added, and the mixture is allowed

[1] *Ibid*

to soak at 25°C. for 0 5 hr. **Approximately one-half of the cooked corn is added to this malt-sprouts mixture** The temperature is then raised to 50°C. and maintained at this level for 0.5 hr. Next the rest of the corn plus an additional 5 cc. of concentrated HCl are added, and the temperature is raised to 62°C. and kept constant until the iodine test for starch is negative. The mash is filtered through burlap bags, and the residue is washed once with warm water The combined filtrate is made up to 30 liters with water and autoclaved at a pressure of 15 lb. per sq. in. for 45 min. The resultant wort should contain 4.5 to 5 per cent of reducing sugar (as glucose) and have a pH of 4.4.

2. *Molasses-salts Medium* —The molasses, calcium phosphate, and sulphuric acid are sterilized together after dissolving them in water. Sterilized urea and ammonium monohydrogen phosphate are added to the cooled solution, and the pH is adjusted to 4.4.

3. *Glucose-salts Medium.*—Solutions of glucose, sodium chloride, magnesium sulphate, calcium chloride, and copper sulphate are autoclaved at a pressure of 15 lb. per sq. in. for 1 hr at a pH of 3.5. A sterilized solution of potassium and ferric pyrophosphates is added to the cooled main mash. If a precipitate forms it is dissolved by adding some sterilized 40 per cent sulphuric acid. A sterilized solution of ammonium chloride and urea is added finally to the main mash, and the pH is adjusted to 4.4 with sterilized 40 per cent sodium hydroxide (NaOH). Although the ratio of carbon to nitrogen in the preceding medium is 25·1, the ratio can be reduced by adding increasing quantities of ammonium chloride and urea

Yields.—Pavcek, Peterson, and Elvehjem have reported the following yields of dry yeast grown on the media indicated:

TABLE 52 —YIELDS OF DRY YEAST[1] FROM VARIOUS MEDIA[2]

Type of yeast	Grain medium, per cent	Molasses-salts medium, per cent	Glucose-salts medium, per cent
Bakers' Yeast A . . .	24 3	34 6	18 0
Bakers' Yeast B	42 5	33 6	34 3
Brewers' Yeast A	34 6	42 7	29 0
Brewers' Yeast A (autoclaved medium)	32 2		
Saccharomyces logos	33 1	28 0	21 4
Willia anomala	21 4	28 6	11 4
Endomyces vernalis	40 9	33 6	30 5

[1] Dry yeast is based on glucose fermented

[2] PAVCEK P L., W H PETERSON, and C A. ELVEHJEM, *Ind Eng Chem*, 30 802 (1938)

Without aeration the yields were of low magnitude—approximately one-tenth of those with aeration.

Active Dry Yeast.—This is a dried bakers' yeast. It is produced by the usual processes and dried to a moisture content of about 8 per cent. Such yeast may be stored for several months without serious loss of its viability. Large quantities were produced for use by the armed forces
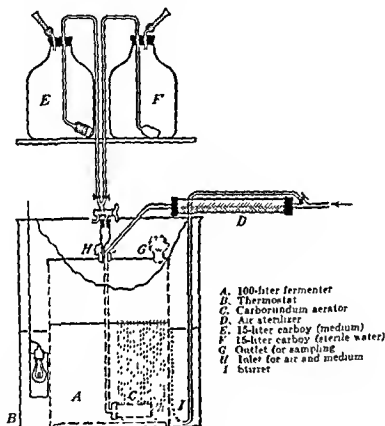
FIG. 37.—Apparatus for growing yeast. [*Courtesy of Profs. P. L. Pavcek, W. H. Peterson and C. A. Elvehjem, Ind. Eng. Chem. 29: 536 (1937).*]

A. 100-liter fermenter
B. Thermostat
C. Carborundum aerator
D. Air sterilizer
E. 15-liter carboy (medium)
F. 15-liter carboy (sterile water)
G. Outlet for sampling
H. Inlet for air and medium
I. Burret

and other agencies during World War II: 7,000,000 lb. in 1943; 13,295,000 lb. in 11 months of the year 1945, and 4,000,000 lb. in 1946.[1]

Florylin Yeast.[2]—This is a dried yeast and is produced from fresh press-yeast, occasionally produced with higher aeration than the usual yeast to encourage glycogen formation.

The press-yeast is put through a flaking machine and spread 1 to 1.5 cm. deep in trays. It is dried by passing air at 30 to 35°C. over the trays for 36 to 48 hr. (or 28°C. for 48 to 50 hr.) The moisture content of the

[1] BORUFF, C. S., and J. M. VAN LANEN, *Ind. Eng. Chem.*, **39**: 934 (1947).
[2] BALLS, A. K., *Ficat Final Report No. 277*, October 3, 1945.

product is 10 per cent. Four grams of the product has the same value as 5 g. of fresh yeast. The product lasts for 8 to 12 months.

Apparatus for Growing Yeast.—Figure 37 shows an apparatus suitable for growing yeast.

FOOD AND FODDER YEASTS

Food yeast is propagated primarily for human consumption as a food constituent. Fodder yeast is produced for animal feeding. Both are produced under similar conditions, except for the raw materials used and the final treatment of the products.

Food yeast is a rich source of protein and vitamins of the B-complex. In the dry state, it usually contains about 50 per cent of protein and when used in the proper proportions with other foods it makes a satisfactory and nutritious supplement to the diet of persons living under conditions where there may be a shortage of animal proteins or vitamins of the B-complex.

The manufacture of fodder yeast is of interest to those desiring to convert waste, surplus, or low-cost carbohydrate materials into products of value for feeding cattle, hogs, or other farm animals. The utilization of waste sulphite liquors for fodder yeast production, for example, results in the extension of animal foods and at the same time aids in the alleviation of stream pollution.

Food yeast was manufactured and used to some extent by the Germans during World War I. Several thousand tons were produced and consumed in Germany during World War II,[1] the raw materials being sulphite liquor or wood hydrolyzates. The Colonial Office of Great Britain undertook the construction of a large plant in Jamaica for producing food yeast early during the latter war [2]. India is much interested in this subject, as are other countries.[3]

General Details of Production.—The following paragraphs contain information of a general nature concerning the production of food and fodder yeasts.

Organisms Used.—The organisms most commonly employed for food or fodder yeast production is a strain of *Torulopsis utilis.* However, other yeasts have been used commercially in Germany, such as *Torula pulcherima, Monilia candida, Candida arborea,* mixed *Torula,* and *Oidium lactis.*[4]

[1] SAEMAN, J. F., E. G. LOCKE, and G. K. DICKERMAN, *Fiat Final Report* No. 499, Nov. 14, 1945
[2] Colonial Food Yeast Ltd., Food Yeast, May, 1944
[3] THAYSEN, A. C., *Food,* p. 116, May, 1945
[4] SAEMAN, LOCKE, and DICKERMAN, *loc. cit.*

A number of other yeasts have been studied, including *Mycotorula lipolytica*, *Hansenula anomala*, *H. suaveolens*, *Candida tropicalis*, *Torulopsis candida*, and *Torula lactosa*.

Torulopsis utilis is particularly desirable on account of its high protein content, its vitamins of the B-complex, and its ability to utilize hexoses, pentoses, acetic acid, and certain other constituents which may be found in the acid hydrolyzates from wood or other cellulosic materials. It normally produces but little alcohol.

There are a number of strains of *T. utilis*. One, known as *T. utilis major*, produces larger cells than the usual strains; while another strain, *T. utilis thermophila* grows well at higher temperatures than most food yeasts.

The biochemistry of *T. utilis* has been studied by Fink and Just. Their findings have been published in a series of articles appearing in *Biochemische Zeitschrift*.[1]

Studies concerning the metabolism of growing *T. utilis* under aerobic conditions have been carried out by Sperber.[2]

REQUIREMENTS OF THE YEAST.—The yeast used for food production should possess the following characteristics: It should be one of high protein and vitamin content. It should grow rapidly, produce high yields, and be palatable. It should be able to grow under the conditions and on the substrates that are available.

ACCLIMATIZATION.—As a rule a faster rate of growth and larger yields of food yeast are obtained from hydrolyzed wood and certain other substrates, if the yeast has become acclimatized to the raw material by being *repeatedly cultured in it.*

SIZE OF INOCULUM.—Usually the concentration of yeast cells in the inoculum is of such magnitude as to give 100,000,000 to 200,000,000 cells in the propagation (fermentation) medium.

Raw Materials.—The raw material used will depend upon a number of factors, the most important of which are availability, cost, and assimilability. In Germany, waste sulphite liquor, particularly from beechwood, was used in six or more plants for food yeast production during World War II. In addition, five plants produced yeast from the acid-hydrolyzed wood obtained by the Bergius and Scholler methods. Molasses and whey are normally available as low-priced carbohydrate-containing materials. Hydrolyzed grains, citrus fruit wastes, wood sugar stillage, and carob beans are other sources. Actually a large variety of raw materials may be used after any necessary pretreatments.

[1] FINK, H., und F. JUST, *Biochem. Zeit.*, **300**: 84 (1938). 303: 1,234,404 (1939), **312**: 390 (1942), etc.
[2] SPERBER, E., *Arkiv Kemi, Mineral Geol.*, **21** (N-03): 1–136 (1945).

Lechner studied the utilization of pentoses[1] and other compounds by *T. utilis*. He found that xylose was readily assimilated,[2] but that rhamnose and glucuronic acid were not utilized.[3]

SUGAR CONCENTRATION —The initial sugar concentration in the propagation tank or container is usually adjusted to 1 per cent or less.[4,5,6] In continuous processes, the concentration of the feed may be much higher.

NITROGEN SOURCES.—Nitrogen may be applied in a number of forms. In Germany, ammonium sulphate and ammonia were used extensively in commercial operations. It was also supplied as diammonium phosphate[7] Urea has been found to be a suitable source of nitrogen by investigators in this country.[5,6]

PHOSPHORUS SOURCES —Diammonium phosphate and superphosphate were used commercially in Germany.[6] Potassium dihydrogen phosphate or superphosphate in equivalent amounts has been used successfully in the United States.[9]

OTHER NUTRIENTS.—In Germany, potassium chloride and magnesium sulphate were used in the production of yeasts from wood sugar. Magnesium chloride has also been used as a source of magnesium on an industrial basis.

pH.—The initial pH is generally adjusted to approximately 4.5, depending upon the raw materials used and the conditions of fermentation. Vincenty and Lewis, Stubbs, and Noble adjusted their media to an initial pH of 4 to 5, Peterson, Snell, and Frazier (1945), to an initial pH of 4 5 to 5.5; and Agarwal, Singh, King, and Peterson (1947), to an initial pH of 4 6

Temperature.—The temperature normally used for food and fodder yeast production is 30°C   Where it is difficult to maintain the temperature at this optimum, special strains of yeasts that grow well at higher temperatures (35 $\pm$ °C) may be used.

Aeration —Provided that other conditions are satisfactory, aeration is probably the most important factor in the successful production of

[1] LECHNER, R , *Angew Chem* , **53**: 163 (1910)
[2] LECHNER, R , *Biochem Zeit* , **304**: 84 (1910)
[3] LECHNER, R , *Biochem Zeit* , **306**: 218 (1910)
[4] VINCENTY, C , Unpublished research report, M I T , Feb 18, 1914
[5] PETERSON, W H , J F SNELL, and W C FRAZIER, *Ind Eng Chem* , **37**: 30 (1915)
[6] AGARWAL, P N , K SINGH, P S KING, and W H PETERSON, *Arch Biochem* , **14** (Nos 1 and 2): 105 (1947)
[7] SAEMAN, LOCKE, and DICKERMAN, *loc cit*
[8] *Ibid*
[9] AGARWAL, SINGH, KING, and PETERSON, *loc cit*

TABLE 53.—YIELDS AND PROTEIN CONTENTS OF YEASTS GROWN IN DIFFERENT MEDIA

Yeast	Medium	Dry yeast yield, per cent	Protein content, per cent	Reference
Torulopsis utilis No. 3	Wood hydrolyzates	35–40*	. . . . . .	PETERSON, SNELL & FRAZIER, *Ind Eng Chem.*, 37 : 30 (1945).
Torulopsis utilis No 3	Wood hydrolysates	30 5–52 6‡	51 9–58 6	HARRIS, SAEMAN, MARQUARDT, HANNAN, & ROGERS, *Ind Eng Chem.*, 40 : 1220 (1948).
Torulopsis utilis No. 3	Sulphite liquor	29 6–39 2‡	51 0–53.7	HARRIS, SAEMAN, MARQUARDT, HANNAN, & ROGERS, *Ind Eng Chem*, 40 : 1220 (1948).
Torulopsis utilis No. 3	Fermentation residue	47 0–52 0‡	50 0	HARRIS, SAEMAN, MARQUARDT, HANNAN, & ROGERS, *Ind Eng Chem.*, 40 : 1220 (1948).
Torulopsis utilis No 3	Wood sugar stillage	53–63‡	52 9	KURTH & CHELDELIN, *Ind. Eng Chem*, 38: 617 (1948).
T. utilis (Anheuser-Busch)	Molasses (cane) Puerto Rican	45 1–48 4‡	33 1–50 8	VINCENNY, M I T Research Report, Feb 15, 1944
T. utilis	Molasses (beet)	53 5–65 3‡	43 7–50 6	AGARWAL, SINGH, KING & PETERSON, *Arch Biochem*, 14 (Nos 1 & 2). 105 (1947).
T. utilis	Molasses	56†	Av. over 50**	LEWIS, STUBBS & NOBLE, *Arch Biochem*, 4 (No. 3), 389 (1944)
T utilis	Fruit juice	53†	Av. over 50**	LEWIS, STUBBS & NOBLE, *Arch Biochem*, 4 (No. 3). 389 (1944).
Torulopsis utilis var major	. . . .	59–62‡	56	THAYSEN & MORRIS, *Nature* 152 (No 3862) 526 (1943)
Candida arborea	Molasses (beet and cane)	55–64‡	36 8–49 4	AGARWAL, SINGH, KING & PETERSON, *Arch Biochem*, 14 (Nos 1 & 2). 105 (1947).
Hansenula anomala	Wood hydrolysates	35–40*	. . . . . .	PETERSON, SNELL & FRAZIER, *Ind Eng Chem*, 37 : 30 (1945)
H suaveolens y-838	Wood sugar stillage	53–63‡	53 4	KURTH & CHELDELIN, *Ind Eng. Chem*, 38: 617 (1948)
Mycotorula lipolytica P-13	Wood hydrolysates	35–40*	. . . . . .	PETERSON, SNELL & FRAZIER, *Ind Eng Chem*, 37 : 30 (1945)
Mycotorula lipolytica P-13	Wood sugar stillage	53–63‡	51 0	KURTH & CHELDELIN, *Ind Eng Chem*, 38: 617 (1948).
Oidium lactis	Molasses (beet and cane)	55 3–60 0‡	31 2–41 6	AGARWAL, SINGH, KING & PETERSON, *Arch Biochem*, 14 (Nos 1 & 2). 105 (1947)
Saccharomyces cerevisiae	Molasses (beet and cane)	42 7–54 3‡	42 5–53 1	AGARWAL, SINGH, KING & PETERSON, *Arch Biochem*, 14 (Nos 1 & 2). 105 (1947).

* Based on total reducing sugars 　‡ Based on sugar consumed 　** In most cases.
† Based on total sugar 　§ Based on sugar in molasses

The yeast suspension is removed continuously. Centrifuges concentrate the liquor to a maximum of 12 per cent. The yeast suspension is concentrated to a 20 per cent cake by a rotary filter. The yeast is then autolyzed and dried on a drum drier; or it may be autolyzed and concentrated (by evaporation) to 30 per cent dry substance and spray dried.

Harris and associates[1] and others have studied food yeast production from sulphite liquors.
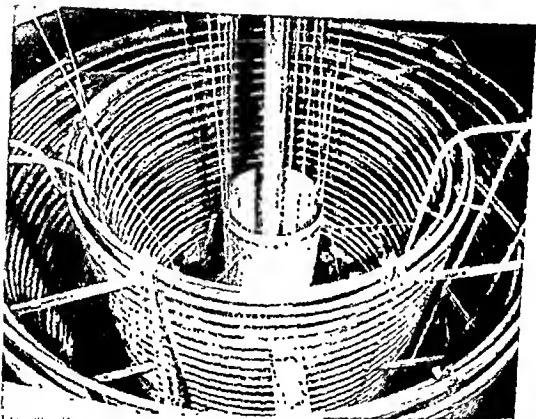
Fig. 38—Cooling coils in fermentor for yeast propagation [Courtesy of J F Saeman, E G Locke, and G. K. Dickerman, Fiat Final Report No. 499, Nov 14, 1945, also E. G. Locke, Pulp & Paper Ind., 20 (No. 1), 20 (1946).]

Fodder Yeast from Sulphite Liquor.—The production of fodder yeast from sulphite liquor on a laboratory scale has been described by Walker and Morgen.[2] The sulphite liquor was neutralized to pH 10.5 with lime, permitted to settle, filtered with the help of filter aid for sludge removal, neutralized to pH 3.5 with sulphuric acid, and filtered. The sugar concentration of different samples of liquor varied from 2 to 2 5 per cent.

[1] HARRIS, E. E., J. F. SAEMAN, R. R. MARQUARDT, M. L. HANNAN, and S. C. ROGERS, Ind Eng. Chem., 40 (No. 7): 1220 (1948).

[2] WALKER, R. D., JR. and R. A. MORGEN, Paper Trade Jour., 123 (No 6) 43 (1946).

Walker and Morgen found that optimum conditions were obtained for fodder yeast production with *Torulopsis utilis* var. *thermophila* when the sulphite liquor contained 2.0 g. diammonium hydrogen phosphate per liter; the pH was 5.75 (5.5 to 6.0); the temperature was 30 to 35°C.; and 150 ml. of air per minute per liter of sulphite liquor (based on the conditions and fermenter used) were employed. Turkey red oil, in a concentration of 0.1 to 0.2 per cent, was effective as an antifoam agent and apparently nontoxic.

During a 15-hr. period, 90 per cent of the reducing sugars were utilized and yeast concentrations of 500 to 700 million cells per milliliter were obtained (as high as 5 per cent by volume).

Inoculum (about 1 per cent) from wort agar slants or from sulphite liquor that was in state of active fermentation were used

Harris, Hannan, and Marquardt[1] described the production of fodder yeast from sulphite liquor. They found that sulphur dioxide and other volatile substances could be removed by steam-stripping or by precipitation with lime. Steam-stripping is the more satisfactory method; however, the equipment used for this must be acid-resistant and is costly. After removal of the sulphur dioxide, the liquor is neutralized with lime to a pH of 4 to 4 5. The resultant precipitate of calcium sulphate, and any pulp fibers, are removed by filtration. In an alternate method, as is used in Germany, the sulphite liquor is first neutralized directly to a pH of 4.1 to precipitate both the sulphites and the sulphates, aerated, and then filtered The necessary nitrogen and phosphate nutrients are added to the liquor, which is then inoculated with *T. utilis* No. 3. Propagation is carried out in the special fermentor described on page 267. Data and results of typical runs are shown in Table 54.

Production from Wood Sugar.—According to Saeman, Locke, and Dickerman,[2] 8,985 tons of yeast were produced in Germany in 1944 by five plants which used sugar obtained by the acid hydrolysis of wood. The wood sugar was produced by the Bergius and Scholler methods.

The process, as carried out at the Regensburg Vogelbusch plant, was as follows· Propagation was conducted in six 250-cu m Vogelbusch fermenters, each of which was equipped with internal cooling coils and a 20 KVA motor which drove the propeller at 8 r.p.m.

The organism used was *Torulopsis utilis*. The inoculum for a fermenter was obtained from a previous fermentation On the basis of the sugar being used, a quantity equivalent to 10 to 20 per cent by weight of dry yeast was employed

[1] HARRIS, E. E, M I. HANNAN, and R R MARQUARDT, *Paper Trade Jour.*, Nov. 27, 1917

[2] SAEMAN, LOCKE, and DICKERMAN, *loc cit.*

TABLE 54.—CONTINUOUS PROPAGATION OF *Torula utilis*[1][2]

Series no.	Rate of feed per hour, liters	Air per pound of dry yeast, cubic feet	Reducing material used, per cent	Yield of dry yeast on sugar used, per cent	Protein content of yeast, per cent
		Wood Hydrolyzate (Sugar content, 4 6 per cent)			
10	2 0	630	92 0	39.0	51 3
14	3 0	205	93 8	49 7	57 5
15	4 0	210	93.4	52.6	56.2
16	4 5	212	93 2	46.5	55.4
17	5 0	203	91 1	43 4	51 5
		Sulphite Waste Liquor from Blow Pit Neutralized with Lime (Sugar content, 1 66 per cent)			
21	2 0	1,040	79 0	40.5	51.6
22	3 0	695	78 0	49 0	51 5
23	4 0	540	75 0	50 0	51 0
24	5 0	550	59 0	50 2	50 0
		Sulphite Waste Liquor, Steam-stripped, Neutralized with Lime (Sugar content, 2.56 per cent)			
748	2	890	77 0	49.5	42 6
752	2	850	74 3	52.0	43.2
765	3	850	75 0	46 1	43.6
770	3	850	73 0	47 4	51.4
		Evaporated Sulphite Waste Liquor, Neutralized with Lime (Sugar content, 12 per cent)			
783	3		68 8	15 4	
784	3		51 8	8 2	
		Evaporated Sulphite Waste Liquor Diluted 1:1 with Water (Sugar content, 6.0 per cent)			
789	2	720	75 0	36 0	43 6
792	2	640	77 6	38 1	
778	3	505	75 9	32 6	
781	3	500	72 6	34 3	37 5
		Magnesia-base Sulphite Waste Liquor (Sugar content, 2.5 per cent)			
L-1	2	1,150	75 4	52 5	
L-6	4	645	71 5	49 0	

[1] Values are averages of a series of experiments
[2] HARRIS, E E., M. L. HANNAN, R R. MARQUARDT, *Paper Trade Jour*, Nov 27 1947

Propagation was carried out at 35°C. For each kilogram of yeast produced, 20 cu.m. of air were supplied.

The suspension of yeasts was centrifuged in a battery of Westphalia centrifuges of the direct-drive type. The cream thus obtained was washed by dilution with water, centrifuged again, concentrated to a 20 per cent cake with a rotary filter, and dried on Oschatz double-drum driers.

The yield of yeast was 45 to 48 per cent based on the sugar

For the manufacture of 500 tons of yeast per month at the Regensburg plant, the following chemical materials were necessary, according to Saeman, Locke, and Dickerman [1]

Material	Tons
Ammonium sulphate	10
Liquid ammonia	75
Potassium chloride	15
Diammonium phosphate	30
Magnesium sulphate	8
Trisodium phosphate	0 5
"Garfett"	7 5
Iupon (another antifoam)	0 5

Peterson, Snell and Frazier [2] studied approximately 150 fermentations of hydrolyzates from 13 different species of wood in which nine types of yeasts were used

These investigators prepared the wood hydrolyzates for fermentation by four different procedures, but the following one was most successful The amounts of the various materials used was based on 5 to 6 per cent reducing sugar Calcium carbonate was added to the hydrolyzate until the pH was 5 0 (approximately 0 75g./5g reducing sugar) Next, 0 05 per cent of sodium sulphite was added. The liquor was then heated to boiling with live steam, cooled and filtered by passage through a pad of Hy-Flo Supercel The nutrient requirements were satisfied by adding 0 06 g of urea and 0 05 g of potassium dihydrogen phosphate for each gram of reducing sugar present The medium was then diluted to the desired concentration

The standard laboratory procedure for yeast propagation by Peterson and his associates was as follows A 6,300-ml amount of the medium was placed in a 5 gal Pyrex bottle It was inoculated with 700 ml of an inoculum which contained 1 g of cells per 100 ml A canvas aerator was placed "well below the surface of the medium" To prevent excessive

[1] Saeman, Locke, and Dickerman, loc cit
[2] Peterson, W H , J F Snell, and W C Frazier, Ind Eng Chem, 37: 30 1945

foaming 10 ml. of Vegifnt Y were added. The Pyrex bottle was introduced into a constant-temperature bath (or incubator) at 30°C. Saturated air at the rate of 20 to 40 liters per min. was passed through the aerator into the medium. The propagation was allowed to continue for 24 hr.

Yields of 35 to 40 per cent of dry yeast, based on the total reducing sugars, were obtained from such yeasts as *T. utilis* No. 3, *Mycotorula lipolytica* P-13, and *Hansenula anomala*. Approximately 90 per cent of the apparent reducing sugar was utilized.

Production from Molasses, Sugar Juice, and Raw Cane Sugar.—Commercial production of food yeast from molasses, sugar juice, and raw cane sugar has been carried out in a plant located at Frome, Jamaica, West Indies. This project was undertaken by the Colonial Office of Great Britain as a result of the need for such a product and of the information and experience obtained at the pilot plant operated at Teddington, England, by the Department of Scientific and Industrial Research. A description of some of the progress made at Teddington and at Jamaica has been furnished by the Colonial Food Yeast, Ltd.,[1] by Thaysen,[2] and by others. The commercial process, as reported by these sources, will be briefly described.

PREPARATION OF WORT.—The wort is made up of the molasses (or sugar juice, or raw cane sugar), nutrients, and water. The molasses is diluted to a concentration just over 10 per cent carbohydrate. If sugar juice, or raw cane sugar, is used, it is similarly adjusted for concentration. It is "sterilized" by heating it to 93.3°C. (200°F.), filtered through plate and frame filters to separate out suspended solids, cooled to 37.8°C. (100°F.) by passage through a heat exchanger, and run into closed mild steel storage tanks.

Ammonium sulphate and superphosphate are dissolved, purified, and adjusted to the desired concentrations in separate equipment. Caustic soda, which is used to adjust the reaction of the fermentation medium, is separately prepared in solution form, also.

The molasses is adjusted to a 10 per cent carbohydrate concentration by dilution with sterile water in a special mixing tank. Ammonium sulphate, superphosphate, and caustic soda are mixed with the molasses in the correct proportions.

THE FERMENTORS.—The fermentors measure 62.5 in. in diameter by 24 ft. in height and are constructed of stainless steel. They are supplied with cooling coils and aeration devices. The latter consists of 32 Aerox aeration candles, each with 0.6 sq. ft. of surface, located at the bottom

[1] Colonial Food Yeast, Ltd., *Food Yeast*, May, 1944
[2] THAYSEN, A. C., *Food*, p 116, May, 1945.

of each fermentor. The pores of the candles do not exceed 10 microns. Thus, the air particles are very small and extremely numerous, increasing the efficiency of aeration. The fermentors are designed for continuous usage for 24 hr. followed by emptying and cleaning

OPERATION —The fermentors are initially filled half-full with dilute wort, containing about 200,000,000 yeast cells per milliliter. After about 3 hr., the rate of multiplication of the cells reaches a maximum wherein it requires approximately 100 min. for a new cell to form from a mother cell The wort is now admitted at a rate such that the concentration of cells will be about 2,000,000,000 per ml. by the time the fermentor is full. From this time on, one-fourth of the total volume of the fermentor is replaced with the same volume of dilute wort each hour. The cell concentration remains at approximately 2,000,000,000 cells per ml.

A measuring device controls the rate at which the wort is fed to the fermentors It may be set at a constant rate or at a logarithmically increasing rate When the fermentors are operated on a continuous basis, another measuring device admits sufficient water to dilute the incoming wort to a concentration of 4 5 per cent sugar.

The pH of the medium is adjusted with caustic soda

The fermented wort is withdrawn from the bottom of the fermentor by means of overflow pipes and collected in a tank.

FINAL STEPS.—The fermented wort is passed through a strainer and then to centrifuges (De Laval-DYK₆). It is separated into a suspension, referred to as yeast cream, and spent wort The latter is discarded to the sewer. The cream is diluted with water and recentrifuged The latter process is repeated After these two washing treatments, the yeast cream is dried on single roll drum driers to a moisture content of about 5 per cent.

The dried yeast is passed under an electric magnet for the purpose of removing any tramp iron. It is then ground, packaged, and stored, or otherwise handled. Action, of course, must be taken to prevent the absorption of moisture by the dried product. The ground, dried preparation weighs 27 to 30 lb per cu ft

CAPACITY OF PLANT.—This is expected to be 12 tons of dried yeast per day of 24 hr.

Production from Wood Sugar Stillage.—Kurth,[1] Kurth and Cheldelin,[2] Harris and associates,[3] and others have studied the production of fodder yeasts from wood sugar stillage on a laboratory basis. Wood sugar

[1] KURTH, E F , *Ind Eng Chem* , **38** (No 2) 204 (1946)

[2] KURTH, E F , and V H CHELDELIN, *Ind Eng Chem* , **38**: 617 (1946)

[3] HARRIS, SAEMAN, MARQUARDT, HANNAN, and ROGERS, *loc cit*

stillage (still waste liquor) is the product remaining after wood sugar hydrolyzates are fermented with yeasts and subsequently distilled and contain pentoses (xylose and arabinose), acids (acetic, formic, etc.), ash, and other constituents. The compositions of the waste liquor obtained from a Douglas fir hydrolyzate and the spent liquors after fermentation are shown in Table 55.

TABLE 55.—ANALYSIS OF LIQUOR BEFORE AND AFTER TORULA GROWTH[1]

	Still waste liquor	Torula spent liquors
Total solids, per cent	3 2	2 40
Reducing sugar (xylose), per cent	0 81	0 20
Reducing sugar after hydrolysis with H_2SO_4, per cent	0.97	0 34
Volatile acid (acetic), per cent	0.24	0.10
Volatile and nonvolatile acid (acetic), per cent	0.78	0.31-0 5
Ash, per cent		
Residue upon combustion	0 05	0 36-0 47
Residue converted to sulphate	1 03	0 53-0 68
Total carbon, per cent	1 42	0.94-1 04
5-day B. O. D	16,400	9,600
pH	5 0	7.5-8 0

[1] KURTH, E. F., Ind. Eng. Chem., 38 (No. 2) 204 (1946).

The yeasts used by Kurth and Cheldelin[1] were Torulopsis utilis No. 3, Mycotorula lipolytica P-13, and Hansenula suaveolens Y-833. They were transferred repeatedly in a wood stillage medium to increase their rate of growth. As inoculums, yeast concentrations of about 100,000,000 cells per ml. were desirable.

The conditions of fermentation were as follows: The medium contained wood sugar stillage from Douglas fir, reinforced by the addition of urea and diammonium hydrogen phosphate in 0 05 per cent concentrations.[1] The pH was maintained between 5.0 and 6.5 with ammonia. After inoculation the liquor was aerated. The rate of growth of the yeast and also of the sugar consumption were affected to the largest extent by aeration. It was discovered that it was most important to use small-sized air bubbles for best results. Table 56 shows the effect of aeration on propagation time and final reducing-sugar content. With suitable aeration the assimilable sugar may be consumed in 18 hr. or less.[2] The rate of sugar consumption was increased as the temperature was increased from 24 to 34°C.

[1] KURTH, CHELDELIN, loc. cit.
[2] KURTH, loc. cit.

TABLE 56.—EFFECT OF AERATION[1]

Aerator	Time, hr	Final reducing sugar, per cent
C-porosity fritted-glass tubes	16	0 30
	19	0 20
Cloth or EC-porosity fritted-glass disks	24	0 20-0 22
4-mm glass tubing	24	0 40-0 49
Shaker flasks	72	0 30

[1] KURTH, E. F., *Ind Eng Chem*, **38** 204 (1946)

Kurth and Cheldelin[1] found that there was but little distinction among the different species of yeasts used in respect to their rate of growth, sugar utilization, yield, and nutritional value. All were able to utilize a large proportion of the residual sugars and acids in the wood sugar stillage. Yields of dry yeasts varied from 53 to 63 per cent on the basis of the sugar consumed

Data concerning the contents of protein, amino acids, and B vitamins of the three yeasts studied by Kurth and Cheldelin are given in Tables 57 and 58.

TABLE 57.—AMINO ACIDS AND PROTEIN CONTENT OF YEASTS
(Percentage of oven-dry weights)[1]

Yeast	Mycotorula	Hansenula	Torula	Brewers'[2]
Total protein	51 0	53 4	52 9	51 8
Amino acid[3]				
Arginine	3 2	2 9	3 1	2 7
Histidine	1 4	1 4	1 5	1 3
Lysine	4 4	4 3	4 4	3 5
Phenylalanine	2 4	2 4	2 3	2 4
Tryptophane	0 3	0 3	0 3	0 8
Threonine	2 5	2 4	2 5	2 8
Leucine	3 7	3 6	3 8	3 7
Isoleucine	3 5	3 7	3 7	3 1
Valine	3 1	3 3	3 3	2 4

[1] KURTH, E. F., and V. H. CHELDELIN, *Ind Eng Chem*, **38** 617 (1946)
[2] Reported by R. J. Block and D. Bolling, *Arch Biochem* **7** 313 (1945) for strain K 103
[3] Values are expressed in terms of the naturally occurring isomer

The production of fodder yeast by a continuous method from wood sugar stillage has been described by Harris and associates[2] (refer to page 263)

[1] KURTH, and CHELDELIN, *loc cit*
[2] HARRIS, SAEMAN, MARQUARDT, HANNAN, ROGERS, *loc cit*.

Fodder Yeast from Citrus-waste Press Juice.—Nolte, von Loesecke, and Pulley[1] described the production of fodder yeast from citrus-waste press juice. This juice is obtained by pressing ground citrus waste (peel, rag, and seeds) that has been limed. It contains 4.08 to 8.58 per cent of total sugars with an average of 6 63 per cent. It is deficient in phosphates and nitrogen.

TABLE 58.—B VITAMIN CONTENT OF YEASTS

	Vitamin content, γ/g. dry wt.[1]		
	Mycotorula	*Hansenula*	*Torula*
Thiamine	5 3	8 5	6 2
Riboflavin	59	54	49
Nicotinic acid	600	590	500
Pantothenic acid		180	130
Biotin	1 8	1 7	1 8
Folic acid	3 1	1 7	2.8
p-Aminobenzoic acid	31	16	17

[1] KORTH, E F., and V H CHELDELIN. *Ind Eng Chem*, 33: 617 (1946)

The juice is prepared for use by screening it, heating it to the boiling temperature, and holding it for 5 min., adding filter aid to it and filtering, diluting it with tap water to adjust the total sugar content to 1 per cent, and cooling to 30°C. Trisodium phosphate and ammonium sulphate are added to the juice in the proportion of 9 per cent based on the total amount of sugar in the batch. Since additional ammonium sulphate is required, it is added during the process at the end of 1 hr. and during the succeeding 3 hr at the rate of 1 75 per cent per hr Sufficient sodium carbonate is added to maintain the pH between 4.4 and 6.5

The nutrient citrus-waste press juice is inoculated with 4 per cent by volume of a 2-day culture of *Torulopsis utilis* in malt wort (which contained 6 per cent of total carbohydrates at the time of inoculation). The medium is aerated vigorously with filtered air and maintained at a temperature of 29 to 30°C. The process is generally completed in 8 hr. The yeast is separated by centrifuging and washed. The yields from citrus-waste juice containing 1 per cent of total sugar (after dilution) are 44.3 to 48.0 per cent of dry yeast (177 to 191.9 per cent of wet yeast containing 25 per cent of solids) based on the total amount of sugar in the juices.

[1] NOLTE, A J., H W. VON LOESECKE, and G. N. PULLEY, *Ind Eng Chem*, 34: 670 (1942).

Production of Fodder Yeast on a Continuous Basis.—The continuous propagation of yeast has been studied by Harris and his fellow researchers.[1] The equipment used (refer to page 267) was a modification of the Waldhof fermentor, which was used in Germany during World War II for the continuous propagation of food yeast from sulphite liquors. It consisted of an open tank of 34-liter fermentation medium capacity; a mechanically driven spinner, 6 in. in diameter, which was operated at a speed of 1,100 r.p.m. and served as a combination aerator and agitator by drawing the fermentation liquor down through a draft tube and forcing it up and around the sides; a proportioning pump to control continuous feeding; and a standpipe, which was slightly higher than the draft tube, for controlling continuous discharge.

The propagation was carried out as follows: Two liters of a solution that contained 100,000,000 yeast cells per ml. (*T. utilis* No. 3) were placed in the fermentor. The spinner was put into operation and then wood sugar hydrolyzate, diluted to a concentration of 2.5 per cent of reducing sugars and containing nutrients, was added at a rate of 2 liters per hr. Air was introduced at the rate of 0.5 cu. ft. per min. The spent medium containing the yeast started to overflow from the fermentor into the standpipe in about 5 hr. The yeast was separated from the liquor by centrifuging and returned to the fermentor until the volume of wet yeast in the fermentor was about 7 per cent, or 1 8 per cent dry yeast. At this point, undiluted wood hydrolyzate, of 4 5 to 5 per cent concentration, was admitted and the rate of air flow was increased to 0 75 cu. ft. per min. The temperature of fermentation was 30 to 32°C and was controlled by cooling coils and an electric heater. No antifoam agent was required due to the type of aerator used. The initial pH of the fermentation medium was 4 7 to 6 6, that of the feed 4 2 to 4.5.

Results of the propagation of *T. utilis* on various types of media and under various conditions are shown in Table 59.

EFFECT OF IRON PRODUCTION OF VITAMINS —Lewis investigated the effect of the iron nutrition of *T* *utilis* on the synthesis of vitamins.[2] He found that iron deficiency caused increased rates of synthesis of thiamin, riboflavin, nicotinic acid, pyridoxin, and pyridoxin isotels per gram of yeast and decreased rates of synthesis of biotin, inositol, p-aminobenzoic acid, and Norit eluate factor isotels. However, no effects on the synthesis of pantothenic acid were observed

Nutritive Value.—The nutritive value of food yeast has been studied by a large number of researchers, particularly in Germany, Great Britain, and this country. These studies have been concerned with such subjects

[1] HARRIS, SAEMAN, MARQUARDT, HANNAN, and ROGERS, *loc cit.*
[2] LEWIS, J C *Arch Biochem*, 4 (No 2)· 217 (1944)

TABLE 59 —CONTINUOUS PROPAGATION OF *Torula utilis* ON WOOD HYDROLYZATE, ON SUGAR RESIDUES FROM ALCOHOLIC FERMENTATION OF WOOD SUGARS, AND ON SULPHITE LIQUOR[1]

Series No.	Rate of feed, liters/hr	Sugar concentration, per cent	pH	Air, cu ft./min.	Air, cu. ft./lb. of yeast	Reducing sugar used, per cent	Dry-yeast yield based on total sugar, per cent	Protein content of yeast, per cent
				Wood hydrolyzate				
9	3 0	4 5	4 7	0 75	415	88 4	36.4	52 0
10	2 0	4 5	5 0	0 75	630	92.0	35.9	52 3
11	4 0	4.5	5 0	0 75	313	86 5	36 0	52 7
12	4 5	4 5	5 0	0 75	275	85.3	36 5	54 4
13	5 0	4 5	5 0	0 75	296	83.1	30.5	52 5
14	3 0	4 6	6 6	0 75	295	93 8	49.7	58 0
15	4 0	4 0	0 0	0 75	210	93 4	52 6	57 3
16	4 5	4 6	0 0	0 75	212	93 2	46 5	56 5
17	5 0	4 6	0 0	0 75	203	91 1	43 4	52 5
18	5 0	4 6	5 5	0 75	208	90 4	42 5	51 9
				Fermentation residues				
19	3 0	0 94	5 4	0 60	1100	60 0	52 0*	50.0
20	4 0	0 91	5 5	0 00	950	55 0	47.0*	50 0
				Sulphite liquor				
21	2 0	1 60	5 0	0 50	1040	79 0	39 2	52 7
22	3 0	1 66	5 0	0 50	695	78 0	39 0	52 5
23	4 0	1 66	5 0	0 50	540	75 0	37 6	52 0
24	5 0	1 66	5 0	0 50	550	59 0	29 6	51.0

[1] HARRIS, E E , J F SAEMAN, R R MARQUARDT, M L HANNAN, and S C ROGERS, *Ind Eng Chem.*, **40** (No 7) 1220 (1948)

* Values corrected to product with 50 per cent protein.

as the digestibility of the yeast in human nutrition; its vitamin content when grown on different media; its protein content; its amino acid content, its value as a food constituent for children and adults and as a feed supplement for hogs, cattle, chickens, rats, and other animals.

Among the reports on these subjects may be mentioned those of Fingerling and Honcamp (1933); Bunger and associates (1934); Fink and Just (1938, 1939), Scheunert and Wagner (1940); Stephenson, Penton, and Korenchevsky (1941); Dirr (1942); Dirr and Von Soden (1942); Hock (1942); Bickel (1942); Thaysen (1943); Thaysen and Morris (1943); Lewis (1944), Lewis, Stubbs, and Noble (1944); Butschek (1944); Wilson

(1944); Colonial Food Yeast, Ltd. (1944); Klose and Fevold (1945); Peters (1945); Kurth and Cheldelin (1946); Agarwal, Singh, King, and Peterson (1947). The subject was reviewed by Carter and Phillips in 1944.

The high protein content of dry yeast has already been referred to (Table 53). There is some indication that yeast protein may be deficient in the amino acid methionine and possibly in cystine, according to Hock and Fink.[1] Klose and Fevold[2] found that torula yeast and brewers' yeast contained inadequate quantities of methionine for the optimum growth rate of chicks. However, a large percentage of animal protein could be replaced satisfactorily with yeast. In spite of these indications, food yeasts may be regarded as valuable protein supplements.

Among the vitamins of the B-complex found in yeast are thiamin, riboflavin, niacin (nicotinic acid), pantothenic acid, biotin, pyridoxine, folic acid, and p-aminobenzoic acid. The thiamin, riboflavin, niacin, and folic acid contents of four different species of yeasts, grown in molasses media and subsequently dried, are shown in Table 60.

TABLE 60—VITAMIN CONTENT OF YEASTS (γ/G. OF DRY YEAST)[1]

Molasses	S terrestris				T utilis				C arborea				O lactis			
	Thiamine	Riboflavin	Niacin	Folic acid	Thiamine	Riboflavin	Niacin	Folic acid	Thiamine	Riboflavin	Niacin	Folic acid	Thiamine	Riboflavin	Niacin	Folic acid
Lansing (beet) Mason City (beet)	37 6	43 8	414 3	21 6	37 5	54 2	520 6	15 2	32 7	69 5	503 1	14 8	20 1	55 0	192 8	7 7
Ojvid (beet)	35 7	50 4	443 3	21 4	36 7	62 0	600 0	10 6	33 1	52 3	492 3	16 0	28 9	9	212 6	5 0
	32 7	45 4	442 8	19 6	36 1	54 8	511 3	11 7	31 3	53 0	512 2	17 6	27 2	42 6	217 5	7 6
Hawaiian (cane blackstrap)	40 8	49 1	568 1	19 1	35 4	58 6	531 4	10 7	33 1	60 0	580 2	15 0	29 0	43 0	242 4	7 8

The media used for the growth of the yeasts were comprised of treated molasses (1 per cent sugar), 0 1 per cent KH_2PO_4, 0 1 per cent urea and 2 ml corn steep liquor   The fermentation was run for 36 hr at 30°C

[1] AGARWAL, P N, R SINGH, P S KING, and W H PETERSON, *Arch Biochem*, 14 (Nos. 1 and 2) 105 (1947)

A number of studies have been made on the vitamin content of *T. utilis*. Lewis and his associates[3] found 18 micrograms of thiamin, 36 micrograms of riboflavin, and 610 micrograms of niacin per gram of yeast (dry basis) produced in molasses media; and 22 micrograms of thiamin, 44 micrograms of riboflavin, and 535 micrograms of niacin per gram of yeast

[1] HOCK, A, and H FINK, *Zeit physiol Chem*, 279: 187 (1943)
[2] KLOSE, A A, and H L FEVOLD, *Jour Nutrition*, 29: 421 (1945)
[3] LEWIS, J C, J J STUBBS, and W M NOBLE, *Arch Biochem*, 4 (No. 3): 389 (1944).

(dry basis) produced in fruit juice media. Kurth and Cheldelin[1] found 6.2 micrograms of thiamin, 49 micrograms of riboflavin, 500 micrograms of niacin, and 2.8 micrograms of folic acid per g. (on a dry basis) in yeast grown in wood sugar stillage. Thaysen[2] reported 23 micrograms of thiamin, 54 micrograms of riboflavin, and 440 to 490 micrograms of niacin per gram of yeast (dry basis) grown in molasses.

Processing of Brewers' Yeast for Use as Food.—Brewers' yeast must be debittered and otherwise treated before use as a food constituent. Reports concerning the processing of brewers' yeast have been presented by Siebel, Weber, and Singruen[3]; Haffenreffer[4]; Burton[5]; MacDonough and Haffenreffer,[6] and others.

In the process described by MacDonough and Haffenreffer,[6] yeast cream (10 per cent yeast on a dry basis) is pumped through a filter into a weighing tank where it is weighed prior to transfer to a debittering tank. The yeast is washed first with water, 10,000 lb. of water being added to 5,000 lb. of yeast slurry. The mixture is separated into a yeast concentrate and waste liquor by passage through an Alpha Laval yeast separator. The yeast is washed next with a caustic soda solution at a pH of about 12.1. Two parts by weight of alkaline water are employed for each part of yeast. The mixture is passed through the separator. The yeast is now washed with a solution containing 0.05 per cent caustic soda (5 lb. of sodium hydroxide in 10,000 lb. of water) and the mixture passed through the separator. Finally the yeast is washed with cold water and concentrated by passage through the separator.

After the debittering treatment, table salt is added to the concentrate in the amount of 2 per cent of the dry weight of the yeast. Likewise sufficient U.S.P. phosphoric acid is added to lower the pH to 5.5 to 5.7.

The yeast preparation may be enriched, if desired, by the addition of a solution containing thiamine, riboflavin, and niacin. The concentrate is stirred uniformly and then dried on a double-drum drier. The final product is pulverized and packaged.

In order to lessen vitamin losses during processing it is essential to use speed and temperatures not above 40°F. These precautions are especially important during the debittering process

[1] Kurth, E. F., and V. H. Cheldelin, *Ind. Eng. Chem.*, **38**: 617 (1946).

[2] Thaysen, A. C., Part III, Food Yeast, *Colonial Food Yeast*, Ltd., May, 1944.

[3] Siebel, R. V., P. J. F. Weber, and E. Singruen, *Modern Brewery Age*, **26** (No. 5): 44 (1941), **26** (No. 6): 46 (1941); **27** (No. 1): 49 (1942), and **27** (No. 2): 74 (1942).

[4] Haffenreffer, T. C., Jr., *Brewers Bull.*, **36** (No. 87): 4 (1943)

[5] Burton, L. V., *Food Inds.*, **15** (No. 11): 66 (1943).

[6] MacDonough, J. V., and T. C. Haffenreffer, Jr., *Wallerstein Labs. Communs.*, **7** (No. 20): 39 (1944).

LABORATORY FERMENTORS FOR YEAST PROPAGATION

A number of laboratory-scale fermentors have been developed for the propagation of yeast. One of these is illustrated in Fig. 37. Another has been described by Feustel and Humfeld.[1] Fermentors designed and

Fig. 39.—Temperature-control equipment of laboratory-scale fermentor designed and built at Forest Products Laboratory   *(Courtesy of E. E. Harris, M. L. Hannan, and R. R. Marquardt, Paper Trade Jour., Nov. 27, 1947.)*

constructed at the Forest Products Laboratory and at the Western Regional Research Laboratory, respectively, are described below.

Forest Products Laboratory Yeast Propagator—Harris, Hannan, and Marquardt[2] constructed a laboratory-scale fermentor (propagator) for the study of yeast production from wood sugars. Some details of the construction of this fermentor are shown in Figs. 39 and 40. The fermentor is constructed preferably of stainless steel in order to resist corrosion and lend itself to easy cleansing.

The propagator (fermentor) consists of several essential parts: an open

[1] FEUSTEL, I. C., and H. HUMFELD, *Jour. Bact.*, **52** (No. 2) 229 (1946).

[2] HARRIS, HANNAN, MARQUARDT, *loc. cit.*

AIR CAP

BALL BEARING

PULLEY

HOLLOW SHAFT

DRAFT TUBE

PRODUCT OVERFLOW

SHAFT BUSHING

AERATION WHEEL

BOTTOM BEARING

FERMENTOR ASSEMBLY

SCALE
0 1 2 3 INCHES

TOP VIEW

SECTION A-A
AERATION WHEEL

SCALE
0   1 INCH

AIR CAP

SCALE
0   1 INCH

VULCANITE SLEEVE

BOTTOM BEARING

SCALE
0   1 INCH

SHAFT BUSHING

SCALE
0   1 INCH
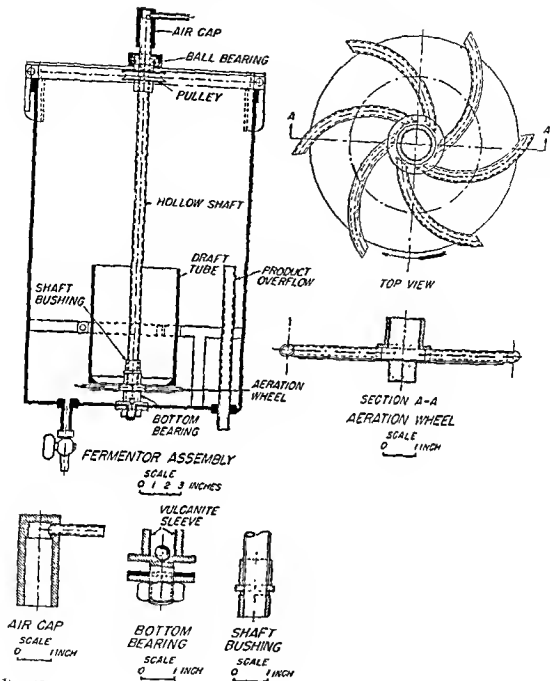
Fig. 40.—Laboratory
Fo
use

tank of 34-liter capacity, with reference to the top of the overflow tube; a motor-driven proportioning pump; an aeration wheel which performs the dual functions of aerating and defoaming; a draft tube; temperature regulating equipment; and a pH controller.

During operation, the fermentation solution is fed continuously by

a proportioning pump into the top of the fermentor at a point next to the shaft of the aeration wheel. A disk on the shaft "just above the liquid" distributes it uniformly in the fermentor. Air enters the hollow shaft of the aeration wheel (which is 6 in. in diameter) at the rate of 1 cu. ft. per min. for each cubic foot of medium and is delivered to the medium through six tubes. The aeration wheel (or spinner) which revolves at the rate of 1,000 r.p.m. pulls the medium out of the draft tube and forces it up the outside liberating the gases as it drops down the draft tube.

Fig. 41 — Fermentor for submerged-culture investigations in operation. [*Courtesy of Dr. Harry Humfeld, Jour. Bact. 54: 692 (1947), and of Western Regional Research Laboratory, Bureau of Agricultural and Industrial Chemistry, U.S. Department of Agriculture.*]

The temperature is regulated by two electric heaters on the outside of the fermentation tank (which are controlled with a bimetallic thermo-switch) and by a cooling tube in the tank through which cold water is circulated as required.

An indicating controller measures and controls the pH of the fermentation medium. A saturated solution of sodium carbonate is added as needed to maintain the pH at 5.3. The feed solution is customarily adjusted to an initial pH of 3.9 to 4.2, since the pH of the fermentation medium increases as the yeast grows.

The yeast and spent liquor pass out through a standpipe.[1]

Western Regional Research Laboratory Fermentor.—Humfeld (1947) described an improved laboratory-scale fermentor for submerged-culture studies. This fermentor has a mechanical foam breaker and can draw air in by the suction produced by the stirring blades. It has a capacity for 10 to 18 liters of medium.
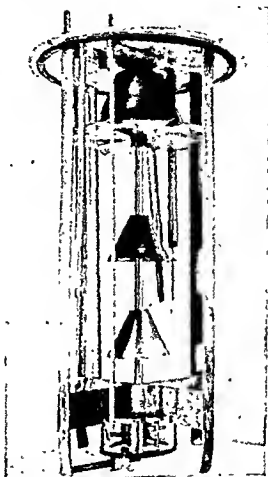
FIG. 42.—Fermentor assembly [*Courtesy of H. Humfeld, Jour. Bact., 54: 689 (1947), and of Western Regional Research Laboratory, Bureau of Agricultural and Industrial Chemistry, U.S Department of Agriculture*]

The tank is a Pyrex glass jar of 12-in. diameter and 24-in. height (Fig. 41). A gasketed stainless-steel cover or lid (2) fits on the top. From this cover an agitation-aeration assembly of stainless steel (Fig 42) is suspended. Plans for the fermentor are presented in Figs. 43 to 46. As will be seen by reference to Fig. 43, the fermentor assembly consists of an air-dispersing unit mounted at the bottom end of the stirring shaft (3); adjustable truncated cones which govern the stirring characteristics

[1] *Ibid.*

to some extent; four metal struts attached to the cover at right angles and connected at the bottom by a metal web to furnish support for the stirring shaft.

The device for aeration and agitation is located at the lower end of the shaft (3). It rotates between stationary circular plates (23 and 24). The upper one (23) is attached to bars (19 and 20); the bottom one is held to the upper plate by studs. The culture medium enters the lower plate through a circular hole (25) Four tubes (28) are attached to the shaft and form area at right angles to it. Vanes (27) are attached to alternate sets of the tubes (Figs 43 and 44).

Air enters through a pipe (21), which extends from the cover to a location directly below the central core of the agitation-aeration device When the shaft is rotated, the vanes (27) cause the medium to flow up through the circular opening (25), bore (30), tubes (28), and out of the casing and past pins (26). The medium also flows through the opening to the casing's interior and out by the pins. As the medium passes the tip of the pipe, air is sucked through the pipe into it.
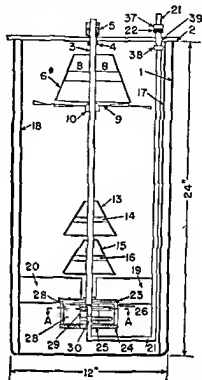
Fig 43 — Side view of fermentor unit (*Courtesy of Western Regional Research Laboratory, Bureau of Agricultural and Industrial Chemistry, U S Department of Agriculture*)

The air passes through bore (30),
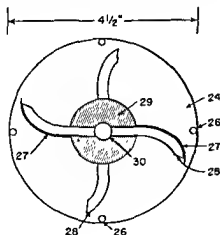
Fig 44 — Top view of stirrer
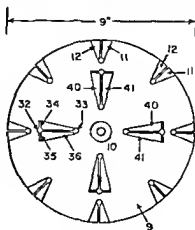
Fig 45 — Top view of foam-breaker disc

tubes (28), and out of the casing. The amount of air used is regulated by raising or lowering the tip of the pipe (21) in respect to the inlet of bore (30).

Cones (13 and 15) are fastened to the shaft by means of webs (14 and 16, respectively) and when rotated mix and agitate the medium.

The foam breaker consists of a cone (6) and a disc (9), located above the normal level of the medium. The disc (9) is secured to the shaft
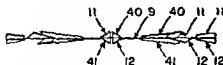
Fig. 46.—Side view of foam-breaker disc. *(Courtesy of Western Regional Research Laboratory, Bureau of Agricultural and Industrial Chemistry, U.S Department of Agriculture.)*

by a collar (10) and contains an interior set of vanes (40 and 41) (Figs. 43 and 44) half of which (40) are turned up and half of which (41) are turned down, and an exterior set of vanes (11 and 12), half of which (11) are turned up and half of which (12) are turned down. When the disc (9) is rotated counterclockwise, the inner vanes (40 and 41) force the medium towards cone (6) and the outer vanes tend to force it towards the side of the vessel or its contents. Thus, the foam is broken effectively. A recent modification of this fermentor consists of a change in the design of the stirrer (Fig. 44), to give a greater capacity for aeration, and the elimination of supporting struts.

The fermentor is provided with a set of pH meter electrodes and with leads protected by removable waterproof tubes. It is also provided with a thermometer well and a sampling tube. The power for operating the fermentor is supplied by a 0.25-hp., ball-bearing, variable-speed electric motor, mounted vertically.

For further details of construction refer to the description furnished by the Western Regional Research Laboratory, U.S Department of Agriculture, Albany, Calif.; for additional information on its operation refer to the paper by Humfeld.[1]

FAT PRODUCTION BY YEASTS AND YEAST-LIKE MICROORGANISMS

Only under conditions of a national emergency would one ordinarily attempt to produce fat from microorganisms, for normally the demands of a country may be met by domestic production from animals and higher plants, or by importations. But when the usual supply becomes inadequate, waste carbohydrate materials may be used for the synthesis of fat by microorganisms.

Fat may be produced by a number of different microorganisms: by yeasts; by yeast-like microorganisms, such as *Oöspora (Oidium) lactis;* and by molds (a discussion of fat production by molds appears in Chap. XXXIV).

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[1] HUMFELD, H., *Jour. Bact.*, 54 (No. 6): 689 (1947).
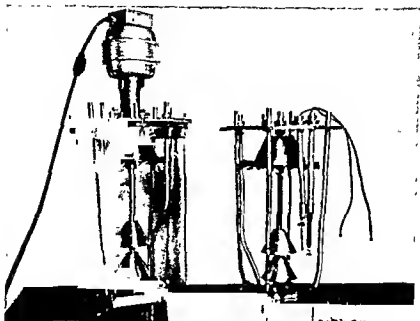
(a)
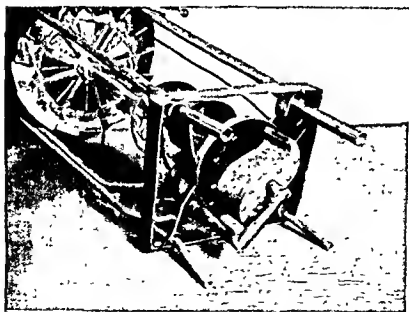
(b)

FIG. 46A.—Modifications of the fermentor *a* (left), fermentor ready for operation,
(right), operating unit removed from container *b*, close-up view of operating unit of
fermentor showing lower end of air-intake pipe leading into center of agitator   Two of the
tubular supports serve as sampling pipes, one as a harvesting tube, and one as a thermom-
eter well   (*Courtesy of Dr Harry Humfeld and the Western Regional Research Laboratory,
Bureau of Agricultural and Industrial Chemistry, U S Department of Agriculture,* 1948)

"Fat" Defined.—The term "fat," as used in this chapter, refers to substances insoluble in water but soluble in ether and other fat solvents. In the literature, one finds that some authors use the term "lipoid" to designate the crude fat obtained from microorganisms. However, in view of the wide use of the word "fat," which is more properly restricted to mean the esters formed by combinations of fatty acids with glycerol, this term will be used throughout the present discussion to include what actually would be classified as lipids, according to Bloor.[1]

Under the term "lipid,"[1] Bloor includes the simple lipids—the fats, oils, and waxes; the compound lipids, such as the phospholipids; and the derived lipids, such as fatty acids, sterols, and alcohols.

The Fat of Yeast.—Some of the first studies concerning the nature of yeast fat were carried out by Nägeli and Loew (1878), Gérard (1895), Gérard and Darcxy (1897), Hinsberg and Roos (1903, 1904) and Sedlmayer (1903). In recent years, amongst others, Smedley-MacLean and coworkers have made notable contributions to the knowledge of this subject.

In yeast fat are found palmitic, oleic, linoleic, and lauric acids; the phospholipids, lecithin and cephalin; ergosterol and zymosterol; and other components. A large proportion of the crude fat is unsaponifiable matter.

Production of Fat by Yeast.—Nägeli and Loew first proved that carbohydrates could be transformed to fat by yeast.[2]

Fat production is a normal function of yeast cells, but the rate of its formation and the quantity of it stored may be increased through the use of certain optimum conditions. Factors of importance in fat production are the use of a medium rich in carbohydrate, oxygenation, and phosphates.

Oxygenation of a medium is essential if greater than normal quantities of fat are desired Nägeli in 1878 showed that the fat content of yeasts and molds could be increased from 5 to 12 per cent when they were grown in well-oxygenated media containing an abundance of carbohydrates and a scarcity of nitrogen.

The addition of alkaline phosphates (Na_2HPO_4 and KH_2PO_4) to a well-oxygenated medium containing 4 per cent glucose caused increased fat storage in a suspension of brewery yeast.[3]

Usually the rate of fat formation was greatest at the beginning of the experiment, diminishing later When the concentration of sugar was

[1] BULL, H. B.; "The Biochemistry of Lipids," John Wiley & Sons, Inc , New York, 1937

[2] NAGELI, C , und O LOEW, Ann , 193: 322 (1878)

[3] SMEDLEY-MACLEAN, I , and D HOFFERT Biochem Jour , 18: 1273 (1924).

increased, the amount of phosphate absorbed by the yeast cells was likewise augmented.

It has been demonstrated that oxygenating a solution containing a suitable sugar increases the amount of fat stored in the suspended yeast. If yeast containing reserve carbohydrate is suspended in oxygenated water, part of the carbohydrate is converted into fat.[1]

Although investigations have been carried out using oxygenated solutions of various simple carbon compounds, thus far only ethyl alcohol and sodium acetate, two-carbon compounds, have produced significant increases in the amount of fat stored by the suspended yeast.[2] These compounds must be used in relatively dilute solution (a 0.5 to 0.6 per cent concentration of ethyl alcohol is satisfactory), because increasing the concentration of either above a low maximum leads to poor results.

The rate at which oxygen is supplied to solutions of ethyl alcohol and sodium acetate determined chiefly the amounts of lipids stored in yeast. When a solution of these compounds is not oxygenated the storage of fat and carbohydrate is not increased.

Alcohol vapor, in the presence of oxygen, leads to a deposition of lipoid material in the cells of brewery yeast and also in *Endomyces vernalis*, according to the researches of Lindner and Unger.[3] Yeast (Frohberg type), when grown in agar in a chamber containing the vapors of ethyl alcohol and oxygen, showed definite increases in fat content.[4] By keeping the moisture content of the yeast low through the use of a drying atmosphere, the highest yields of fat were obtained in the presence of alcohol vapor. Substitution of ethyl alcohol with propyl alcohol in control experiments yielded negative results

The following table indicates the average increases in the fat content of samples of yeast incubated in oxygenated water and in oxygenated solutions of N/14 acetates[5]

TABLE 61 — AVERAGE INCREASES IN FAT CONTENT OF YEAST IN VARIOUS SOLUTIONS

Solution	Percentage Increase in Fat Content
Water	41
Potassium acetate	180
Sodium acetate	160
Magnesium acetate	118
Calcium acetate	100

[1] SMEDLEY-MACLEAN, I , *Ergeb Enzymforsch*, **5**: 285 (1936).
[2] SMEDLEY-MACLEAN and HOFFERT, *op. cit*, **20**: 343 (1926)
[3] LINDNER, P, und UNGER, *Wochschr. Brau*, **36**: 188 (1919); LINDNER, P, *Zeit angew Chem.*, **35**: 110 (1922)
[4] HALDEN, H, *Biochem Zeit*, **225**: 249 (1931)
[5] MACLEOD, L. D., and I SMEDLEY-MACLEAN, *Biochem Jour*, **32**: 1571 (1938).

When phosphate is added to a 0.6 per cent solution of sodium acetate, there is no increase in the quantity of fat produced from the acetate.

Fat is produced from sodium lactate and sodium pyruvate in oxygenated solutions containing yeast, according to Smedley-MacLean and coworkers. Shaking the solutions of these compounds increased the quantity of fat and carbohydrate stored. Lactic acid, or lactate, usually yields carbon dioxide and small amounts of volatile acids in addition to the stored products. Pyruvate is not so effective as lactate in forming storage compounds.

No increases in the lipid content of yeast, not equally well obtained by oxygenating a suspension of yeast in water, were observed by oxygenating solutions of the sodium salts of citric, crotonic, fumaric, gluconic, levulic, maleic, or succinic acids; acetoin; 2:3-butyleneglycol; or methylethyl ketone.[1]

The addition of calcium or magnesium ions to an oxygenated solution of glucose significantly decreased the quantity of lipids usually stored in yeast. Likewise, the addition of these ions to oxygenated solutions of acetates caused a decrease in the amount of lipid material ordinarily formed from the acetates.[1]

Fat Production by Endomyces Vernalis.—Considerable research was undertaken by Lindner and his associates at the Institut für Gärungsgewerbe of Berlin at the time of World War I in an effort to produce fat from *Endomyces vernalis* on a successful economic basis[2]

E. vernalis grows as a mat or skin over the surface of a liquid medium. For fat production, oxygen is essential. However, agitation of the medium is not beneficial.

Several carbohydrates are assimilated but not fermented. Since fat storage is sought, this is a desirable feature of the organism. Molasses, cellulose waste, hydrolyzed wood, and other media containing an assimilable source of carbohydrate may be used as raw materials for fat production.

Waste sulphite liquor, fortified with nitrogenous substances and the necessary salts, is a favorable raw material. Nitrogen-containing substances may include yeast water, ammonium salts, urea, urine, molasses slop, extracts of grains, or other products. Potassium chloride, primary potassium phosphate, and magnesium sulphate may be used as salts.

An abundance of a suitable carbohydrate is essential for the maximum production of fat

The optimum temperature for growth is 15 to 20°C., although a temperature as low as 10°C. may be used.

[1] *Ibid.*

[2] FINK, H., H. HAEHN, und W. HOFFBURGER, *Chem. Ztg.*, 61: 689, 723, 744 (1937).

In the production of fat by *E. vernalis*, two phases may be recognized in the incubation period: the phase during which the principal growth of the organism occurs (2 to 3 days under optimum conditions) and the phase of principal fat formation, which generally requires 6 to 8 days in addition. The phase of growth has been designated as the "protein generation" by Lindner. During this phase, the organism may be satisfactorily used for inoculation purposes. For growth the organism may be cultivated in a medium rich in nitrogen but poor in carbohydrates, but for fat production ("fat generation") a medium rich in a carbohydrate is essential. Thus is illustrated the difference between the optimum conditions for growth and optimum conditions for the production of a desirable end product.

Though many efforts were made to find satisfactory methods for producing fat from *E. vernalis*, only two methods were carried out on a large scale, the floor process and the pan process

1. *The Floor Process.*—In this process, inert materials such as chopped straw or coarse sawdust was washed, impregnated with the nutrient medium, and sterilized. The impregnated inert material was spread in thin loose heaps over the floor and inoculated with a fine suspension of *E. vernalis* The heaps were turned several times a day and occasionally sprayed with water. At 11°C., fat production was completed in approximately 12 days; at 20°C , in 8 to 10 days. The fungus was dried at a temperature of not above 50°C.

The following data (Lindner) are taken from a test in which the inert material was impregnated with a 25 per cent molasses solution and inoculated with *E vernalis*·

TABLE 62—DATA ON FAT PRODUCTION BY *Endomyces vernalis*

Amount of sugar used	12 5 kg
Sugar used in CO_2 production	4 16 kg
Sugar left for fat formation	8 34 kg
Theoretical yield of fat	3 33 kg
Actual yield of fat	0 95 kg
Percentage of theoretical yield	28 5
Yield on basis of sugar used, per cent	7 6

The amount of sugar converted to fat by this process was small. Furthermore, difficulties were encountered in separating the fungus from the inert material, frequent attention was necessary during growth of the organism, and infection, especially with *Torula*, was prevalent There was, however, adequate surface exposure.

2. *The Pan Process*—In this process, which was carried out on a large scale, the organism was grown in large flat pans containing a shallow layer (1 to 2 cm. in depth) of a sterilized nutrient solution of sugar.

In order to conserve space and to facilitate handling, the pans were placed one above the other in frames. No covers were placed over the pans.

When the mat, or growth, on the surface of the solution in the pan became well-developed, a large part of the culture solution was carefully run off and replaced with fresh nutrient sugar solution. After the maximum fat formation (usually in 7 to 8 days), the spent culture solution was drawn off and the mat carefully washed by repeated underlayerings with water. The mats thus obtained, rich in fat, were used as a paste. Such paste was designated, for example, as "Evernal" or "Myceta." The protein content of such pastes was valuable.

In this process sugar utilization was nearly complete, but the necessary use of much hand labor increased the costs, while infection by yeasts, molds, or bacteria was a very serious matter in some cases.

Recovery of Fat.—Fat may be obtained from *E. vernalis* by one of several methods: by chemical means, by extraction with ether, and by an autolytic process. In the chemical method the fungus mat is treated with warm dilute hydrochloric acid to decompose the cells. The fat, obtained as neutral fat, may be used for food. The efficiency of the process is high.

The cells are ground with sand to disintegrate them before extraction with ether. Fat so obtained is used industrially. The efficiency of this process is not high.

Self-digestion is permitted at approximately 50°C. for 2 to 3 days in the autolytic process. The fat is recovered from the autolyzate.

Preservation of the Fat.—The fat keeps well if oxygen is completely excluded.

Fat Production by Oöspora (Oïdium).—In the year 1926, Chapman isolated a species of *Oöspora (Oïdium)* from a sewer blocked by the growth of this organism. Culture of this species in a nutrient solution led to the formation of a thick film within 2 days, which contained 10 per cent fat and 50 per cent protein. The flavor and odor of the film resembled cream cheese.[1]

Out of 50 strains of *Oöspora (Oïdium) lactis* examined—10 of which produced considerable quantities of fat—Fink, Haeseler, and Schmidt[2] selected two, strains *A* and *B*, which reproduced well and produced good yields of fat. Of these two strains, strain *A* was preferred, for it was less sensitive to higher temperatures and it reproduced more uniformly.

[1] RAMSBOTTOM, J., *Brit. Assoc. Advancement Sci. (Annual Report)*, Sept. 10, 1936

[2] FINK, H., G. HAESELER, und M. SCHMIDT, *Zeit. Spiritusind.*, 60: 74, 76–77, 81–82 (1937), FINK, H., H. HAEHN, und W. HOERBURGER, *Chem. Ztg.*, 61: 744–747 (1937).

Oöspora lactis, or, as it has been commonly designated in the past, *Oïdium lactis*, is frequently found in Camembert cheese[1] and in some butters and may be a cause of spoilage of cream cheeses. It is quite resistant to heat and infection by other microorganisms. This attribute of growing in a medium that has become infected by other microorganisms and, at the same time, giving good results makes the mold especially desirable.

Optimum Conditions for Fat Production.—Since *Oöspora lactis* occurs naturally in dairy products, whey is an excellent source of raw material. Whey may be used alone or enriched with other nutrient substances. Urea, ammonium sulphate, or ammonium acetate are good sources of nitrogen, while primary potassium phosphate (KH_2PO_4) and magnesium sulphate are excellent sources of potassium, phosphate, magnesium, and sulphur.

As an illustration of the nature of the nutrient medium and the results obtained when *Oöspora lactis* is grown in Jena flasks, the following experiment is quoted.[2] The nutrient solution contained 2 liters of whey, 2 g. of ammonium sulphate, 1 g. of primary potassium phosphate, and 0.5 g. of magnesium sulphate. In each of a series of Jena flasks, 250-cc. portions were placed. Data for the experiment are shown in Table 63.

An examination of this table shows that under the conditions of the experiment the maximum yield of crude fat was obtained in 6 days.

Other experiments by Fink, Haessler, and Schmidt have indicated that the shallow pan or dish was superior to the Jena flask as a container.

The optimum temperature for fat production was 25 to 30°C.

A sugar concentration of 4 to 6 per cent was satisfactory for both strains *A* and *B*.

Using strain *A*, Fink and his coworkers obtained yields of 12.5 to 14.34 g. of crude fat within 5 days from 100 g. of sugar in whey enriched with ammonium sulphate, potassium chloride, and magnesium sulphate. These yields, according to Fink and his associates, were better than those obtained by Geffers[3] from pure wheys, calculated on the sugar utilized, when using strains of *Oöspora wallroth*, strains similar to *Oöspora lactis*, and were secured in a shorter period of time. However, Geffers obtained yields as high as 50 per cent of the dry weight of *Oöspora wallroth*, when using lactose.

Production from Straw and Oat Hulls.—A German process for the production of fat by *Oöspora* (*Oïdium*) *lactis* from the sugars derived from

[1] Hammer, B. W., "Dairy Bacteriology," 2d ed., John Wiley & Sons, Inc., New York, 1938.

[2] Fink, Haehn, und Hoffmeister, *loc. cit.*

[3] Geffers, H., *Arch. Mikrobiol.*, **8**: 66–98 (1937).

straw (by a modified Scholler process) and from oat hulls has been described by Balls.[1] Two types of apparatus were used. One of these consisted of a rotating drum, made by stretching canvas over a wooden frame about 4 ft. in diameter, which was open at the ends near the central shaft. The nutrient solution was placed on the inside of the drum. In order to inoculate the medium, the inside surface of the canvas was sprayed or painted with a culture of *O. lactis.* The drum was rotated about two times a minute during operation. The second type of apparatus consisted of a row of sheets of canvas hung about 3 in. apart on a wooden frame. One side of the canvas was painted with a culture of the

TABLE 63.—DATA ON FAT PRODUCTION BY *Oöspora lactis*[1]

Age, days	Yield in dry material, grams	Nitrogen, per cent of dry substance	Crude protein extraction, per cent of dry substance	Crude fat, per cent of dry substance	Total yield of crude fat, grams	pH of the nutrient solution
2	1 777	5.40	33 75	7.5	0.133	5 0
3	3 238	3 32	20 75	10 8	0.349	(6 5)
4	3 968	3 15	19 69	13 4	0 531	5 0
5	4 771	2 98	18 62	16 7	0.796	5 0
6	5 729	2 81	17 56	22.5	1.290	7.1
7	6 147	2 76	17 25	22 0	1.352	7 7
12	5 886	2 85	17 81	19 6	1 152	8 3
16	5 710	3 15	16 69	16 6	0.948	8.1

[1] FINK, H., D. HAENN, und W. HOENBURGER, *Chem. Zig.*, 61: 744–747 (1937).

organism   Nutrient solution was pumped through nozzles onto the other side of the canvas and trickled down to a tray below. It was pumped continuously from the trays to the canvas until the sugar was nearly consumed. Filtered air was circulated between the canvas sheets. The process was complete in about 5 days at room temperature. The yield was said to be about 20 per cent of fat on a dry basis. For further details, the reader is referred to Balls' report.

Fat Production by Rhodotorula Gracilis.—The production of fat from *Rhodotorula gracilis* has been described by Enebo and associates.[2,3] Fat contents of 50 to 60 per cent (on a dry basis) were obtained in noncon-

[1] BALLS, A K., *Fiat Final Report No. 371*, Oct 10, 1945

[2] ENEBO, L , M ELANDER, F BERG, H. LUNDIN, R NILSSON, and K MYRBÄCH, *Iva*, 6: 1 (1944)

[3] ENEBO, L , L G ANDERSON, and H LUNDIN, *Arch Biochem*, 11 (No. 3): 383 (1946)

tinuous cultivation with about 51 liters of substrate. The fat coefficient (number of grams of newly formed fat per 100 g of sugar consumed) was 16 to 18; the generation time, 15 to 20 hr.; and the protein content, 12 to 13 per cent, under these conditions.

Conditions of Production.—The medium used by Enebo and his fellow workers[1] in most of their experiments had the following composition:

Constituent	Grams/liter
Invert sugar	40 0
(NH₄)₂SO₄	1 0
K₂HPO₄	1 0
MgSO₄·7H₂O	1 0
NaCl	0 5
CaCl₂·6H₂O	0 5
FeCl₃·6H₂O	0 005
Beer wort (20 per cent)	25 ml
pH (with H₂SO₄) to	4 5

The yeast was grown in a cylinder of acid-resistant steel, which was 25 cm. in diameter and 225 cm. high, and which was provided with an aerating device at the bottom and a rotating foam-suppressor.

The temperature of incubation was 27 to 29°C.

Observations.—In one typical experiment, the time of cultivation was 68 hr.; the volume of nutrient solution, 47 liters; the volume of the seed culture, 5 5 liters containing 46.0 g of yeast on a dry basis; the newly formed yeast (dry substances), 547 g.; the added sugar, 1,880 g ; the yield of yeast, 29.1 per cent; the content of crude fat in dry yeast substance, 56 0 per cent; the newly formed fat, 326 g ; the fat coefficient, 17.3, the degree of reproduction, 12.9; the number of generations, 3 69; the generation time, 18 4 hr.; the nitrogen content of the yeast, 2 10 per cent; and the ash content of the yeast, 3 5 per cent.

Figure 47 shows the relationship between sugar content, quantity of yeast, and fat content as functions of time

Fat Production by a Soil Yeast.—Starkey[2] has described lipid production by a yeast isolated from the soil, which appeared to be closely related to *Torulopsis lipofera*. He found that the lipid production of the cells was largest when the yeasts were grown on a nitrogen-deficient carbohydrate medium Large amounts of lipid were produced in an aerated solution that contained 3 per cent glucose, 0.05 per cent (NH₄)₂SO₄, and 0.01 per cent yeast extract The conversion of glucose to lipid varied from 10 to 14 per cent; the lipid content of cells (on a dry basis), from 50 to 63 per cent.

[1] ENEBO, L, M ELANDER, F BERG, H LUNDIN, R NILSSON and K MYRBÄCK, *Iva.* 6: 1 (1944)

[2] STARKEY, R L, *Jour Bact.* 51: 33 (1946)
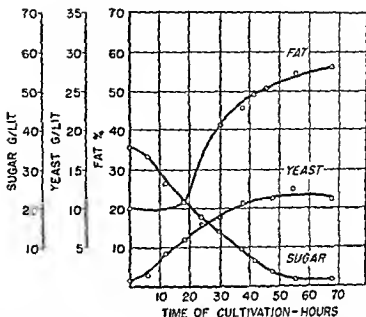
FIG. 47 —Sugar content, quantity of yeast, and fat content as functions of time. [*Courtesy of L Enebo, L G Anderson, and H. Lundin, Arch, Biochem , 11 (No 3). 383 (1946)*]

THE VITAMINS OF YEAST

Vitamin production in yeasts varies qualitatively and quantitatively, as has already been indicated in the discussion on food yeasts. Although thiamin, riboflavin, nicotinic acid, pantothenic acid, biotin, pyridoxine, folic acid, and p-aminobenzoic acid are produced by yeasts in general, many strains form little or none of some of these vitamins. However, certain strains of yeasts are particularly good sources of thiamin, riboflavin, or others of the vitamins when propagated under optimum conditions.

Millions of pounds of yeast, especially brewers' yeast, have been used to enrich foods and in pharmaceutical products. Huge amounts have been employed in livestock feed.

Vitamin B_1.—Vitamin B_1 (thiamin, or aneurin), the antiberiberi vitamin, whose structural formula follows,[1] is produced in varying amounts by different yeasts, depending largely upon the nature of the medium in which they are grown.
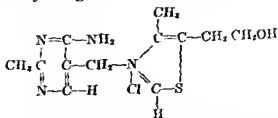
[1] ADDINALL, C. R , "The Story of Vitamin B_1" Merck & Co., Inc , Rahway, N J , 1937.

According to the investigations of Pavcek, Peterson, and Elvehjem, the vitamin B_1 content of most of the yeasts grown on the same medium under similar conditions was approximately the same. The vitamin B_1 content varied widely on different media, however.

The vitamin B_1 content of the yeasts listed in the following table was approximately 10 I.U. (International Units) per g of dry yeast for the grain medium, with the exception of *Endomyces vernalis*, which yielded approximately 7 I.U per g. For the molasses-salts medium, the vitamin B_1 content varied from 3 to 4 I.U. (*E. vernalis*, excepted); for the glucose-salts medium, the vitamin B_1 content varied from 2.5 to 3.3 I.U. (omitting *E. vernalis*).

TABLE 64 —APPROXIMATE AMOUNT OF VITAMIN B_1 PER GRAM DRY YEAST[1]

	Grain medium, I U	Molasses-salts medium, I.U	Glucose-salts medium, I U
Bakers' yeast A	<10	<3	3
Bakers' yeast B	10	3	<3 3
Brewers' yeast A	10	<3	<3 3
Brewers' yeast A (medium auto-claved)	10		
Saccharomyces logos	10	<3	<2 5
Willia anomala	<10	4	<3 3
Endomyces vernalis	7	5	7

[1] Data from the investigations of Pavcek and his coworkers

The superiority of the grain medium from the point of view of vitamin B_1 production is evident. Both the grain and the molasses media contained vitamin B_1 before inoculation with yeast, while the glucose-salts medium contained none.

The indications are that yeast will abstract vitamin B_1 from the medium, when it is thus available, in preference to synthesizing it [1] The addition of vitamin B_1 crystals, yeast concentrates of B_1, nucleic acid, or liver extract increased the yields of the vitamin produced by bakers' yeast, strain B, from the glucose-salts medium (Nucleic acid, which contains no vitamin B_1, was used because it was believed that it might function as a precursor of the vitamin [2])

Apparently yeast is able to resynthesize vitamin B_1 from its decomposition products, for the destruction of the vitamin in grain medium by prolonged heating at pH 6 did not decrease the yield of the vitamin [2]

[1] FISCHER, A M , *Brewers Digest*, **13**: 37 (No 10) (1938)
[2] PAVCEK, P L , W H PETERSON, and C A ELVEHJEM Ind Eng Chem , **29**: 536 (1937)

The vitamin B_1 potency of yeast is increased when aeration is not used, but the yield of yeast is small under such conditions.

Riboflavin Synthesis by Yeasts.—Riboflavin (vitamin B_2, vitamin G, lactoflavin) has the empirical formula $C_{17}H_{20}N_4O_6$ and the structural formula indicated below:
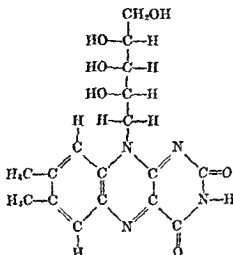
When in the form of a crystalline yellow-orange powder, it is bitter in taste and practically odorless.

Rogosa[1] has shown that lactose-fermenting yeasts have the ability to synthesize riboflavin when grown on a riboflavin-free medium. The results of some of his findings are summarized in Table 65 which follows.

TABLE 65.—QUANTITY OF RIBOFLAVIN SYNTHESIZED BY SOME LACTOSE-FERMENTING YEASTS[1]

Culture	Riboflavin synthesized, γ/ml. of culture
Saccharomyces anamensis No. 145	0 08
Type F No. 03	0 08
Monilia pseudotropicalis (Castellani) No. 32	0 06
Mycotorula lactis No. 130	0 08
Saccharomyces lactis No. 131	0 09
Torulopsis kefir No. 149	0 10
Zygosaccharomyces lactis No. 90	0 06
Z. lactis No. 27	0 10
Torula sphaerica No. 13	0 09
T. lactosa No. 168	0 11
T. cremoris No. 2	0 06
Saccharomyces fragilis No. 15	0 09

[1] Rogosa, M., Jour. Bact., 45 (No. 5): 459-460 (1942).

[1] Rogosa, M., Jour. Bact., 45 (No. 5): 459-460 (1942).

Production by Ashbya gossypii.—The formation of riboflavin by *Ashbya gossypii* was recognized by Guilliermond, Fontaine, and Raffy.[1] Production of this vitamin by a variant of *A. gossypii* has been studied by Wickerham and his associates.[2] In preliminary experiments, they used two media. One of these contained 0.3 per cent powdered yeast extract, 0.5 per cent peptone, and 2 per cent cerelose. The pH of this medium was 6.8 to 7.0. The second medium was of the same composition except that it contained in addition 0.3 per cent of Stimuflav, a commercial preparation of dried distillers' solubles. The pH of the latter medium was adjusted with KOH to 6.8 to 7.0.

Aeration, obtained by passing air through the medium or by agitation on a Ross-Kershaw shaker, increased the yields of riboflavin over unaerated cultures. An aeration rate of about 75 ml. of air per 400 ml. of medium per minute appeared to be optimum. Incubation was at 26 to 28°C. Foaming was prevented by the use of 3 drops per culture of a 1:1,000 concentration of octadecanol in lard oil.

Yields of 381 γ of riboflavin per milliter were obtained in 8 days from *A. gossypii*, NRRL Y-1056, in a 2 per cent cerelose-yeast extract medium aerated at the rate of 75 ml. of air per minute per culture. The yield of riboflavin was increased from about 70 γ per ml. to 120 to 130 γ per ml. in 4 days by the addition of 0.3 per cent of Stimuflav and 0.1 per cent of calcium carbonate to the yeast extract medium and by aeration at the rate of 75 ml. of air per minute per culture.

Wickerham and his associates found that sucrose or maltose (but not lactose) could be substituted for cerelose; that sugar concentrations greater than 2 per cent gave poorer yields; and that it was not necessary to remove iron from the medium in order to obtain good yields.

Production by Eremothecium ashbyii.—Riboflavin is produced industrially from *Eremothecium ashbyii* a yeast-like organism belonging to the Ascomycetes. The latter was described by Guilliermond (1935), who, together with Fontaine and Raffy (1935), noted that it produced a yellow pigment related to the flavin group. Mirimanoff and Raffy (1938) extracted the crystalline flavin from *E. ashbyii*. It was found by Schopfer (1944) that *E. ashbyii* required biotin, inositol, thiamin, and some constituents of peptone for growth and that riboflavin production was stimulated by peptone.

Riboflavin production has been described in patents. In one method described by Rudert (1945), it is produced by *E. ashbyii* from sub-

[1] GUILLIERMOND, A., M. FONTAINE, and A. RAFFY, *Compt. rend. acad. sci. Paris,* 201: 1077 (1935).

[2] WICKERHAM, L. J., M. H. FLICKINGER, and R. M. JOHNSTON, *Arch. Biochem.,* 9: 95 (1946).

stantially carbohydrate-free media. Based on the total weight of nutrients, the medium contains 10 to 90 per cent of proteinaceous material, a metabolizable lipid, and nutrients such as peptone or a combination of salts (0.05 per cent KH$_2$PO$_4$, 0.07 per cent MgSO$_4$·7H$_2$O, 0.10 per cent NaCl, and 0.001 per cent FeSO$_4$·7H$_2$O). Examples of the media and lipids used, and yields obtained, are shown in the following table:

TABLE 66a.—RIBOFLAVIN PRODUCTION FROM DIFFERENT MEDIA[1]

Lipid source	Grams/ 100 ml	Peptone 2.4 g./100 ml (micrograms/ml.)	Salts and egg albumen (0 6 g /100 ml.) (micrograms/ml.)	Initial pH	Final pH
Corn oil	0 6	...	178	6 5	7 1
Mazola oil	0 6	..	113	6.5	
Olive oil	0 6	110	...	6.5	7.1
Peanut oil	0 6	.	107	6.5	6.7
Cocoa oil	0 6	134	...	6.5	7.1

[1] RUDERT, F J., U S Patent 2,374,503, Apr. 24, 1945

In carrying out production, the following procedure is illustrative of Rudert's invention: The media are adjusted to an initial pH of 5.5 to 7 5 and dispensed in containers to give a depth of 0.5 in., sterilized at 20 lb. pressure for 45 min., cooled to 30°C., and inoculated with 0.7 per cent of an active culture of *E ashbyii*. During production the temperature is maintained between 20 and 34°C. and the medium is aerated with 1 5 to 2 cu. ft. of sterile air per minute per square foot of mash surface. At the end of 50 to 90 hr the conversion is complete and the final pH is usually 6.5 to 7.2. The dried residues contain 200 to 6,000 micrograms per g of riboflavin.

In a patented method, described by Piersma (1946), *E ashbyii* is grown on a medium containing animal proteinaceous material, a carbohydrate and malt extract. The proteinaceous material may be liver, pancreas, spleen, lung, liver, or other substance of related nature. The carbohydrate source may be glucose, maltose, sucrose, molasses, corn syrup, etc., or a combination of several of these. The concentration employed may be 1 to 5 per cent proteinaceous material, 0.25 to 1.5 per cent carbohydrate, and 0.5 to 5 per cent of malt extract (optimum 1.5 to 2 0 per cent). The pH of the medium is initially 5.5, and the temperature 27 to 30°C. Oxygen is supplied by aeration. The yield of riboflavin is generally 150 to 500 γ per ml. of medium; for example, in a mash containing 4 per cent liver cake (residue from aqueous extract of liver), 0 5 per

cent sucrose, and 1.75 per cent malt extract, the yield of riboflavin was 304 γ per ml. in 88 hr.

Production by Candida Species.—The production of riboflavin by species of the genus *Candida* has been studied by Burkholder (1943, 1944), Tanner, Vojnovich, and Van Lanen (1945); Tanner and Van Lanen (1947); and others.

Burkholder (1943) found that *C. guilliermondia* grew and produced riboflavin satisfactorily in media containing dextrose, mannose, levulose, or sucrose. Asparagine and glycine were suitable and relatively inexpensive sources of nitrogen for riboflavin production.

Burkholder (1944) patented a process for producing riboflavin from *C. guilliermondia* (A.T.C.C 9058). A medium of the following composition proved to be "highly satisfactory":

	Grams/ Liter		Parts per Million
KH$_2$PO$_4$	0 5	Boron[1]	0 01
MgSO$_4$·7H$_2$O	0 5	Manganese[1]	0 01
CaCl$_2$·2H$_2$O	0 3	Zinc[1]	0 07
(NH$_4$)$_2$SO$_4$	2 0	Copper[1]	0 01
KI	0 1	Molybdenum[1]	0 01
Asparagine	2 0	Iron[1]	0 01
Dextrose	20 0	Biotin[2]	1 microgram

[1] Supplied as chlorides or soluble salts
[2] Supplied as methyl ester or crude concentrate

Most suitable results were obtained when the pH was adjusted to 5 0 to 6.0, although the range of 5 to 7 was satisfactory. The temperature was 30°C. Increased yields of riboflavin were obtained by adding small amounts of sterile potassium cyanide or cyanide gas to the medium after vigorous fermentation was obtained, usually after 24 hr. The fermentation time was 6 to 7 days.

Tanner and Van Lanen[1] have patented a method for producing riboflavin from *Candida flareri* The method briefly consists of growing under aerobic conditions at 30°C. for about 7 days *C. flareri*, or other suitable *Candida* species, in a medium containing a fermentable sugar, an assimilable source of nitrogen, non-iron inorganic salts, biotin, and less than 10 3 micrograms of iron per 100 ml.

The preferred species of *Candida* are *C flareri* and *C. guilliermondia*, but other suitable species may be used.

It is essential to use great care in controlling the iron content of the medium, for iron exerts a critical influence on the yield A low iron content may be obtained in several ways, among which are the following. (1)

[1] TANNER, F W , JR , and J M VAN LANEN, U S Patent 2,421,003, July 15, 1947.

by the treatment of the medium by its passage through an ion exchange apparatus for the removal of iron (for example, with the use of Nalcite MX, Nalcite iron remover, or Nalcite AX[1]); (2) by the selection of the ingredients of known low iron content; or (3) by the treatment of the medium with chloroform and 8-hydroxyquinoline. In the latter procedure, the medium (about 200 ml. of basal) is shaken in a separatory funnel with a mixture of 10 mg. of 8-hydroxyquinoline in about 2 ml. of chloroform. The mixture is allowed to stand until the chloroform separates out, after which the latter is withdrawn. Additional chloroform is added, the mixture shaken and allowed to settle, and then the chloroform separated out. This procedure is repeated, 8-hydroxyquinoline being added in alternative extractions, until the chloroform layer that settles out appears to be colorless, which indicates that not more than 0.3 microgram of iron per 100 ml. remains in the medium.

Various media may be used as long as they conform to the general specifications outlined above and contain less than 10.3 micrograms of iron per 100 ml. Tanner and Van Lanen, in several experiments, used a basal medium that contained dissolved in 200 ml. of water, 40 g. of glucose, 2.0 g. of asparagine, 2.0 g. of urea, 0.5 g. of KH_2PO_4, 0.5 g. of $MgSO_4 \cdot 7H_2O$, and 1.0 microgram of biotin (free acid). This basal medium was treated with chloroform and 8-hydroxyquinoline to remove most of the iron and made up to 1 liter with triply distilled water in a container that would not contribute iron. One hundred-milliliter portions of the medium were dispensed in 500-ml. Erlenmeyer flasks, sterilized at 126°C for 15 min , cooled, and inoculated with a suspension of yeast cells which had been washed previously with triply distilled water in a centrifuge to remove loosely adherent iron.

As an alternate procedure, the glucose is dissolved in 1 liter of water and then treated by passage through an ion exchange column containing Nalcite MX (or other suitable iron remover). The other ingredients mentioned above are then added, and the pH is adjusted to 5.0 (4.5 to 5 5) with NaOH or other alkali. The medium is sterilized, cooled, and inoculated as described above.

Tanner and Van Lanen state in their patent that various substitutions may be made in the basal medium. Fermentable sugars, such as arabinose, dextrose, levulose, maltose, mannitol, sucrose, or xylose may be used. Ammonium chloride, ammonium nitrate, ammonium sulphate, or ammonium phosphate may be employed as the source of inorganic nitrogen, while asparagine, glutamic acid, or hydrolyzed casein may be employed as the source of organic nitrogen. Phosphorus may be supplied as phosphoric acid, dipotassium hydrogen phosphate, or ammonium

[1] TANNER, F W , JR , and J. M. VAN LANEN, U S Patent 2,424,003, July 15, 1947

phosphate; sulphur, as magnesium sulphate, sodium sulphate, or potassium sulphate; potassium, as dipotassium hydrogen phosphate or potassium sulphate; and magnesium, as magnesium sulphate, magnesium chloride, or magnesium nitrate. Biotin may be supplied purified as the free acid or the methyl ester, or in its natural forms

Agitation and aeration increase the yields and shorten the fermentation time.

Maximum yields were obtained from the basal medium described above when the iron concentration was between 0.5 and 1.3 microgram per 100 ml. Table 66b indicates the influence of the iron concentration on the yield at the end of 7 days.

TABLE 66b—EFFECT OF IRON CONCENTRATION ON RIBOFLAVIN PRODUCTION BY SELECTED SPECIES OF *Candida*[1]

Culture	Iron concentration, micrograms/100 cc.	Dry wt. of yeast, g /100 cc.	Dry wt of cell-free residue, g /100 cc	Riboflavin, micrograms/cc.
	0 0– 0 3	0 27	0 45	108 0
Candida guilliermondia	0 5– 0 8	0 57	0 67	123 0
(NRRL 488)	1 0– 1 3	0 66	0 56	120 0
	10 0–10 3	0 89	0 26	7 2
	50 0–50 3	0 92	0 19	3 2
	0 0– 0 3	0 21	0 37	107 0
C guilliermondia	0 5– 0 8	0 67	0 33	125 0
(NRRL 324).	1 0– 1 3	0 61	0 33	157 0
	10 0–10 3	0 89	0 35	16 5
	50 0–50 3	0 82	0 19	10 6
	0 0– 0 3	0 42	1 30	195 0
C flareri	0 5– 0 8	0 49	0 75	216 0
(NRRL 245)	1 0– 1 3	0 55	0 72	216 0
	10 0–10 3	1 12	0 28	8 9
	50 0–50 3	1 31	0 52	4 1

[1] TANNER F W , JR and J M VAN LANEN, U S Patent 2 421 001 July 15 1947.

Recovery of Riboflavin.—Riboflavin may be recovered from production substrates by a variety of procedures, many of them patented Keresztesy (1944) patented a procedure for extracting riboflavin with butanol, followed by the use of other solvents, such as petroleum ether and acetone. McMillan (1945) patented a chemical precipitation method in which a soluble reducing agent and a finely divided diatomaceous earth were used Hines (January, 1945) described a method wherein ribo-

flavin was adsorbed on fuller's earth, silica jel, or other adsorbent, and eluted with an aldehyde, ketone, or alcoholic solution of an organic base.

TABLE 66e.—SOME VITAMINS PRODUCED BY YEASTS
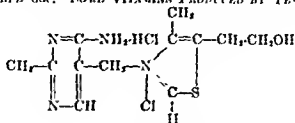
Thiamin hydrochloride (vitamin B₁)
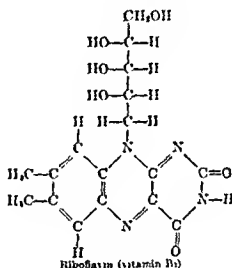
Riboflavin (vitamin B₂)
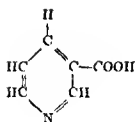
Nicotinic acid
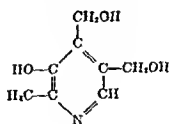
Pyridoxine (vitamin B₆)
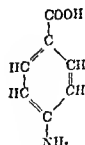
p-Aminobenzoic acid

Pantothenic acid
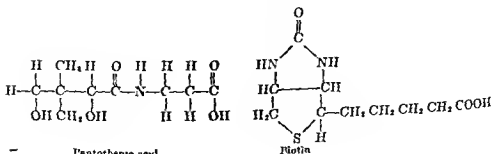
Biotin

Another procedure by Hines (October, 1945) related to the conversion of riboflavin to a less soluble form by the action of reducing bacteria, such as *Streptococcus faecalis*. Dale (1947) has patented a method for securing crystalline riboflavin from the precipitates produced by the reduction of this vitamin to a less soluble form by either reducing bacteria or chemical reducing agents

Ergosterol and Vitamin D.—Yeasts contain ergosterol, a substance which, when irradiated, forms vitamin D. The quantity of ergosterol produced by different yeasts varies. Irradiated yeast may be produced in the fresh or dry condition. Ergosterol may be extracted from the yeasts and then irradiated.

The vitamin D content of milk is increased by feeding irradiated dry yeast to cows.[1]

Bunker and Harris have reappraised vitamin D milks [2]

Since vitamin and ergosterol production by yeast are factors subject to variation, each lot of yeast must be bio-assayed in order to determine the exact amounts of these substances present

Yeast may be dried under carefully controlled conditions and still maintain an effective vitamin content

The feeding of yeast to persons suffering with pellagra has brought definite relief [3] Dried yeast is a rich source of the pellagra-preventing factor [4]

INVERTASE

The enzyme invertase is produced by growing yeasts. Invertase is used by confectioners, bakers, and sirup manufacturers It converts sucrose to glucose and fructose by inversion, thus making possible sugar content without crystallization taking place.

The subject of invertase has been reviewed by Neuberg and Roberts (1946)

References on Yeast: Its Manufacture, Vitamins, and Uses

ADDINALL, C R : "The Story of Vitamin B₁," Merck & Co , Inc , Rahway, N J , 1937
BALLS, A K , and J B BROWN Studies in Yeast Metabolism, I, Introduction, *Jour. Biol Chem* , 62: 789–821 (1925), II Carbon Dioxide and Alcohol, *Jour Biol. Chem* , 62: 823–836 (1925)

[1] WACHTEL, M , *Munch med Wochschr*, 76: 1513 (1929), STEENBOCK, H , E B
HART, F HANNING, and G C HUMPHREY, *Jour Biol Chem* , 88: 197 (1930)
[2] BUNKER, J W M., and R S HARRIS, *New Engl Jour Med* , 219: 9 (1938).
[3] GOLDBERGER, J , and W. F TANNER, *U S Pub Health Rpts* , 40: 54 (1925);
GOLDBERGER, J., G A. WHEELER, and W F TANNER, *U S Pub Health Rpts* , 40:
927 (1925)
[4] *U S Dept Agr* , *Misc Pub* 275, June, 1937

BROCKMANN, M. C., and T. J. B. STIER: Steady State Fermentation by Yeast in a Growth Medium, *Jour. Cellular Comp. Physiol.*, 29 (No 1): 1–14 (1947)

BUNKER, J W M., and R S. HARRIS: A Reappraisal of Vitamin D Milks, *New Engl. Jour Med*, 219: 9 (1938).

CASTOR, J. G. B., and T J. B. STIER: Apparatus for Continuous Yeast Culture, *Science*, 106 (No. 2741): 43 (1947).

CHRZASZCZ, T., and J JANICKI: Recent Advances in the Fermentation Industries, *Chem and Ind*, 55: 884 (1936)

DANIEL, E P., and H. E. MUNSELL: Vitamin Content of Foods, *U.S. Dept. Agr*, *Misc. Pub.* 275, June, 1937

EWESON, E W.: Yeast from Wood, *Chem. Ind*, 38: 573 (1936).

FEUSTEL, I C., and H. HUMFELD: A New Laboratory Fermenter for Yeast Production Investigations, *Jour. Bact*, 52 (No 2): 229–235 (1946).

FISCHER, A M : Yeast and Factors Determining Its Vitamin Potency, *Brewers Digest*, 13: 37 (No. 10) (1938).

FREY, C N · History and Development of the Yeast Industry, *Ind. Eng. Chem*, 22: 1154 (1930)

———, G W KIRBY, and A SCHULTZ: Yeast Physiology, Manufacture and Uses, *Ing Eng Chem*, 28: 879 (1936)

GOLDBERGER, J , and W. F. TANNER: A Study of the Pellagra-preventive Action of Dried Beans, Casein, Dried Milk, and Brewers' Yeast, with a Consideration of the Essential Preventive Factors Involved, *U S Pub Health Rpts*, 40: 54 (1925)

———, G A WHEELER, and W. F. TANNER: Yeast in the Treatment of Pellagra and Black Tongue, *U. S. Pub Health Rpts*, 40: 927 (1925).

HUMFELD, H . An Improved Laboratory-scale Fermenter for Submerged Culture Investigations, *Jour. Bact*, 54 (No. 6): 689–696 (1947).

NEUBERG, C , and I S ROBERTS: Invertase, *Scientific Report Series No 4*, Sugar Research Foundation, Inc , New York, September, 1946.

PAVCEK, P L , W H. PETERSON, and C A. ELVEHJEM: Effect of Growth Conditions on Yield and Vitamin B₁ of Yeast, *Ind Eng Chem*, 29: 536 (1937)

———, ———, and ———: Factors Affecting the Vitamin B₁ Content of Yeast, *Ind Eng. Chem*, 30: 802 (1938).

PETERSON, W H , J T SKINNER, and F. M. STRONG: "Elements of Food Biochemistry," Prentice-Hall, Inc., New York, 1943.

RAMSBOTTOM, J : The Uses of Fungi, *Brit. Assoc Advancement Sci*, Annual Report, 1936.

SCHOLLER, H: Die Chemie im Dienste der nationalen Rohstoffversorgung. Die Gewinnung von Zucker, Spiritus und Futterhefe aus Holz als Rohstoff, *Chem. Ztg*, 60: 293 (1936).

SLATOR, A · Yeast Crops and Factors Which Determine Them, *Jour. Chem. Soc*, 119: 115 (1921)

STEENBOCK, H , E B. HART, F. HANNING, and G. C. HUMPHREY *Jour Biol Chem*, 88: 197 (1930).

STEPHANY, C. D , and H W VON LOESECKE: "Selected References on Yeast," War Food Administration, Washington, D.C., January, 1945.

UNGER, E D , W H STARK, R E. SCALF, and P KOLACHOV: Continuous Aerobic Process for Distiller's Yeast *Ind Eng. Chem*, 34: 1402–1405 (1942)

VAN LANEN, J M , H P. BROQUIST, M J. JOHNSON, 1 L BALDWIN, and W. H. PETERSON, Synthesis of Vitamin B₁ by Yeast, *Ind. Eng Chem*, Ind Ed., 34 (No. 10): 1244 (1942)

WACHTEL, M.: *Münch. med. Wochschr.*, **76**: 1513 (1929).

WHITE, J.: A Critical Survey of Methods for the Determination of Yeast Quantity, *Am. Brewer*, 80 (No. 8): 40 (1947)

Some Patents

BENNETT, W. G.: Manufacture of Yeast, U S Patent 2,059,980, Nov. 3, 1936

BERTEL, R : Manufacture of Dry Yeast, U S Patent 1,859,250, May, 17 1932

BROWNE, H H.: Manufacture of Yeast, U S Patent 2,166,339, July 18, 1939.

BUHRIG, W H , A SCHULTZ, and C N FREY: Compressed Yeast Product, U.S Patent 1,970,275, Aug 14, 1934

DARÁNYI, S C.: Manufacture of Yeast, U.S Patent 2,035,048, Mar 24, 1936

EPSTEIN, A K.: Process of Growing Yeast, U S Patent 1,858,488, May 17, 1932

FRESHEL, C P : Process for the Treatment of Yeast and the Product Produced Thereby, U S. Patent 1,981,225, Nov 20, 1934.

HEIJKENSKJOLD, O. W. Method of Manufacturing Yeast, U S Patent 1,881,557, Oct 11, 1932.

IRVIN, R R., and M. W. MEAD, JR.: Process for the Production of Yeast, U S Patent 2,183,570, Dec 19, 1939

JANSEN, S : Manufacture of Yeast, U S Patent 2,056,576, Oct 6, 1936

JELLINCK, E . Process of Producing Yeast without Alcohol, U S Patent 1,920,395, Aug 1, 1933

KIRBY, G W , and C N FREY Manufacture of Yeast, U.S. Patent 2,029,572, Feb 4, 1936

KITZMEYER, E L : Process of Manufacturing Yeast, U.S. Patent 2,097,292, Oct. 26, 1937

LOCKEY, J . Aeration Apparatus, U S Patent 2,151,126, Mar. 21, 1939

MEYER, E A Manufacture of Yeast, U S Patent 1,938,081, Dec 5, 1933

MIZOBATA, K Method of Preparing Dried Yeast, U S Patent 1,934,941, Nov 14, 1933

MOSKOVITS, M : Manufacture of Yeast of High Enzymatic Activity, U S Patent 1,962,831, June 12, 1934.

NILSSON, M , and N S HARRISON, Process for Producing Yeast, U S Patent 1,449,127, Mar 20, 1923

POLLAK, A : Manufacture of Yeast, U S Patent 2,094,023, Sept 28, 1937

REICH, G T . Art of Purifying Yeast, U S Patent 2,031,668, Feb 25, 1936

RILEY, H : Process for Treating Yeast, U S Patent 1,909,011, May 16, 1933

———: Preserved and Dried Yeast, U S Patent 1,991,629, Feb 19, 1935

SAK, S . Method of Controlling Yeast Propagation, U S Patent 1,884,272, Oct. 25, 1932

——— Yeast Propagation Controlling Method, U S Patent 1,891,841, Dec 20, 1932

SCHULTZ, A : Manufacture of Yeast, U S Patent 2,029,592, Feb 4, 1936

———: Manufacture of Yeast, U S Patent 2,079,634, Mar 11, 1937

——— and C N FREY Manufacture of Yeast, U S Patent 1,893,152, Jan 3, 1933

SHAVER, A , and ——— Manufacture of Yeast, U S Patent 1,910,265, May 23, 1933

THOMAS, C A , and C A HOCHWALT. Fermentation Process and Product U S Patent 2,138,431, Nov. 29, 1938

VAN THIEL, E : Apparatus for the Manufacture of Yeast, U S Patent 2 043,940, June 9, 1936

VOGELBUSCH, W : Apparatus and Process for Aerating Wort in Yeast Production, U S. Patent 2,121,458, June 21, 1938

WHITE, J. R.: Yeast Composition and Process of Making the Same, U.S. Patent 1,974,937, Sept. 25, 1934

————: Dry Yeast and Method of Preparing the Same, U.S. Patent 1,974,938, Sept. 25, 1934

WILLIAMS, R. J.: Yeast Culture in Bread Making and Yeast Propagation, U.S. Patent 2,147,762, Feb 21, 1939

————: Method of Producing Yeast, U S Patent 2,174,543, Oct. 3, 1939.

WROTEN, J. F: Process for Manufacturing Yeast, U.S. Patent 1,917,283, July 11, 1933.

Some References Concerning Food and Fodder Yeasts

AGARWAL, P. N, K. SINGH, P. S KIND, and W. H. PETERSON: Yields and Vitamin Content of Food Yeasts Grown on Different Kinds of Molasses, Arch. Biochem, 14 (Nos 1 and 2): 105–115 (1947).

BALLS, A K.: Yeast Production from Wood Sugars by the Bergins Process, Fiat Final Report No. 493, Jan. 3, 1946.

BICKEL, A : Zur Kenntnis des ernahrungsphysiologischen Wertes der Hefeeiweisses, Biochem. Zeit, 310: 355–377 (1912)

BLOCK, R J, and D. BOLLING. The Amino Acids Yielded by Various Yeasts after Hydrolysis of the Fat-Free Material—A Comparative Investigation, Arch Biochem, 7: 313–321 (1945)

BUTSCHEK, G : Chem. Ztg, 12. 230 (1944)

CARTER, H E, and C E PHILLIPS. The Nutritive Value of Yeast Proteins, Fed. Proc, 3: 123 (1944)

Colonial Food Yeast Limited "Food Yeast—A Venture in Practical Nutrition," Brook House, Park Lane, London, W. I., 1944.

DILL, D B, Manufacture of Torula Food Yeast, Zellstoffabrik Waldhof, Office of the Publication Board, Dept of Commerce, Report 201, Washington, D C.

DIRR, K : Über den Wert der Wuchshefen für die menschliche Ernahrung II Mitteilung' über die biologische Brauchbarkeit der Bergin-Holzzuckertrockenhefe, Biochem Zeit, 312: 233–251 (1912).

——— und O. v Soden. Über den Wert der Wuchshefen für die menschliche Ernahrung 1. Mitteilung Aminosäuregehalt von Bergin-Holzzuckertrockenhefe, Biochem. Zeit, 309: 329–336 (1941).

——— und ———: IV. Über die Lipoide der Wuchshefen, Biochem. Zeit, 312: 263–276 (1942)R

ENEBO, L., H LUNDIN, und K MYRBACH: Österr. Chem. Ztg, 45: 9–15, 34–38 (1943)

FINK, H, und F JUST: Zur Biochemie der Torula utilis. II Mitteilung: Über die zusammensetzung der Hefesubstanz, insbesondere des Hefeeiweisses, Biochem Zeit, 300: 84–88 (1938).

——— und ———. Zur Biochemie der Torula utilis IV. Mitteilung: Der Gesamtschwefel, Glutathion- und Cystingehalt verschiedener Hefen, Biochem. Zeit, 303: 234–241 (1939)

——— und ———. Zur Biochemie der Torula utilis VII. Mitteilung: Zugleich Ergänzung zu der Mitteilung von K. Dirr und O v. Soden "Über den Wert Wuchshefen für die menschliche Ernahrung I Mitteilung, Aminosäuregehalt von Bergin-Holzzuckertrockenhefe, Biochem Zeit, 312: 390–393 (1942).

HARRIS, E E, M L HANNAN, and R R. MARQUARDT: Production of Fodder Yeast, Paper Trade Jour, Nov 27, 1947.

———, J. F. SAEMAN, R. R. MARQUARDT, M L HANNAN, and S. C. ROGERS: Fodder Yeast from Wood Hydrolyzates and Still Residues, *Ind. Eng. Chem.*, 40 (No. 7): 1220–1223 (1948).

HARRIS, G C M : Food Yeast. The Mycologists Contribution to Post War Nutrition, *Rev. Applied Mycol.*, 25 : 132 (1945)

FLOCK, A.: Über den biologischen Ergänzungswert verschiedener Nahrungsproteine, I. Mitteilung: Der Ersatz von tierischem Eiweiss durch Hefeeiweiss, *Biochem. Zeit.*, 311: 385–401 (1942)

JUST, F, und H FINK Zur Biochemie der *Torula utilis.* III. Mitteilung. Vergleichende Untersuchungen uber den Phosphatgehalt von *Torula utilis* und Bierhefe, *Biochem Zeit*, 303: 1–9 (1939)

KLOSE, A A, and H L FEVOLD: Nutritional Value of Yeast Protein to the Rat and the Chick, *Jour. Nutrition*, 29 : 421–430 (1945).

KURTH, E F. Yeasts from Wood Sugar Stillage, *Ind Eng. Chem*, 38 : 204–207 (1946).

——— and V. H CHELDELIN Feeding Yeasts from Wood Sugar Stillage, *Ind Eng Chem*, 38 : 617–619 (1946)

LECHNER, R Über die Verhefung der Pentosen, *Angew. Chem.*, 53 : 163–167 (1940)

———: Über die Ausnutzung der Pentosen bei der biologischen Eiweissynthese V. Mitteilung Zuchtungen von *Torula utilis* in Xylose und in Galaktose, *Biochem Zeit*, 304: 84–89 (1940)

——— Über die Ausnutzung der Pentosen bei der biologischen Eiweiss synthese VI Mitteilung· Zuchtung von *Torula utilis* in Arabinose, Rhamnose und Glucuronsaure, *Biochem Zeit*, 306: 218–223 (1940)

LEWIS, J C : Relationship of Iron Nutrition to the Synthesis of Vitamins by *Torulopsis utilis*, *Arch Biochem*, 4 (No 2) 217–228 (1944)

———, J. J. STUBBS, and W M NOBLE· Vitamin Synthesis by Torula Yeast, *Arch Biochem*, 4 (No 3) 389–401 (1944)

LOCKE, E J.: Yeast Production in Germany from Sulfite Waste Liquors, *Pulp & Paper Ind*, 20 (No 1) 20–26 (1946)

MUKHERJEE, J K : A Review of the Present Knowledge on the Production of Food Yeast, Unpublished report at the Massachusetts Institute of Technology, January 1947

Northeastern Wood Utilization Council· Wood Yeast for Animal Feed, *Bull* 12, New Haven 6, Conn , November, 1946

PAVCEK, P L , Wood-Sugar Yeast Manufacture, *PB Report* 4292

PETERS, R A Food Yeast A Summary of Its Nutritive Value, *Med Res Council (G B)*, *War Memo* 16 (1945)

PETERSON, W H , J F SNELL, and W C FRAZIER Fodder Yeast from Wood Sugar, *Ind Eng Chem*, 37: 30–35 (1945)

ROSENQUIST, O : How Sweden Produces Yeast for Nutritional Purposes, *Food Inds*, 16 (No 6) 443–444, 487 (1944)

SAEMAN, J F, E G LOCKE, and G K DICKERMAN The Production of Wood Sugar in Germany and Its Conversion to Yeast and Alcohol, *Fiat Final Report* 499, Nov 14, 1945

SCHEUNERT, A , und K H WAGNER· Weitere Untersuchungen über den Vitamin B₁- und B₂-Gehalt von Holzzuckertrockenhefen, *Biochem Zeit*, 303: 329–334 (1939)

———, ———, H FINK, und J KREBS Die Synthese von Vitamin B₁ und B₂ (Komplex) durch die Hefe *Torula utilis Biochem Zeit*, 302: 1–11 (1939)

SKOOG, F . Food Yeast Production and Utilization in Germany, *PB Report* 2041.

Sperber, E.: Studies in the Metabolism of Growing *Torulopsis utilis* under Aerobic Conditions, *Arkiv Kemi, Mineral. Geol.*, 21 (No. 3): 1–136 (1915).

Thaysen, A C.: Value of Micro-organisms in Nutrition (Food Yeast), *Nature*, 151 (No. 3832): 406–408 (1913).

——: Production of Food Yeast, *Food*, pp. 116–119 (May, 1915).

—— and M. Morris: Preparation of a Giant Strain of *Torulopsis utilis*, *Nature*, 152 (No. 3862): 526–528 (1913).

Von Soden, O, und K Dinn: III. Verdaulichkeit in vitro von verschiedenen Hefen im Vergleich zu anderen Eiweisstragen der menschlichen Ernahrung, *Biochem Zeit*, 312 : 252–262 (1942)

Walken, R D , Jr., und R A. Morgen: Protein Feed from Sulphite Waste Liquor, *Paper Trade Jour*, 123 (No 6): 43–47 (Aug. 8, 1946).

Wilson, F. A.: Yeast in Nutrition, *Intern. Sugar Jour.*, 46: 154–156 (1944).

Some References on Brewers' Yeast

Dunlop, S G.: Dried Yeast in Army Rations, *Modern Brewery Age*, 33 (No. 3): 24 (1945).

Fairbrother, T. H . Food Yeast, *Food Manuf*, 19 (No. 9): 309 (1944).

Haffenreffer, T. C., Jr.: Practical Application of Yeast Conservation and Processing, *Brewers Bull* , 36 (No. 87). 4 (1943).   Refer also to *Food Inds.*, 15 (No. 11): 66 (1943).

Rehn, J B · Microbiological Examination and Evaluation of Brewers' Yeast, *Wallerstein Labs. Communs* , 6 (No. 10): 191–197 (1943).

Siebel, R. V., P J. F. Weber, and E. Singheen: Functions and Properties of Vitamins in Waste Brewers' Yeast, *Modern Brewery Age*, 26 (No. 5): 44 (1941).

——, ——, and ——: Production Phases of Waste Brewers' Yeast, *Modern Brewery Age*, 26 (No 6)* 46 (1941).

——, ——, and ——: Spray Drying Brewers' Yeast, *Modern Brewery Age*, 27 (No. 1): 49 (1942).

——, ——, and ——: Drum Drying Brewers' Yeast, *Modern Brewery Age*, 27 (No 2): 74 (1942)

References on Fat Production by Yeasts and Yeast-like Microorganisms

Balls, A. K.: Production of Fat by *Oidium lactis*, *Fiat Final Report* 371, Oct. 10, 1945.

Beijerinck, M. W.: Levures chromogenes: nouvelle réaction biologique du fer, *Arch Neerland physiol*, 2 : 609 (1918).

Bull, H B . "The Biochemistry of Lipids," John Wiley & Sons, Inc , New York, 1937.

Enebo, L , L G. Anderson, and H. Lundin: Microbiological Fat Synthesis by Means of Rhodotorula Yeast, *Arch Biochem* , 11 (No. 3): 383–395 (1946).

Fink, H , H. Haehn, and W. Hoenburger: Über die Versuche zur Fettgewinnung mittels Mikroorganismen mit besonderer Berucksichtigung der Arbeiten des Institut fur Garungsgewerbe, *Chem. Ztg.*, 61: 659–663, 723–726, 744–747 (1937).

——, G. Haeseler, und M Schmidt: *Zeit. Spiritus ind.*, 60: 74, 76, 81 (1937)

Geffers, H : *Arch Mikrobiol* , 8: 66–98 (1937).

Guilliermond, A · "The Yeasts," translated and revised by F W Tanner, John Wiley & Sons, Inc , New York, 1920

Haehn, H , und W. Kintrof: Über den chemischen Mechanismus bei der Fettbildung in der lebenden Zelle, *Ber* , 56 : 439 (1923)

Hammer, B. W.: "Dairy Bacteriology," 3d ed., John Wiley & Sons, Inc., New York, 1948.

Hinsberg, O., and E. Roos: Über einige Bestandteile der Hefe, *Zeit. physiol. Chem.*, 38: 1 (1903).

—— und ——: Nachtrag zu der Abhandlung über einige Bestandteile der Hefe, *Zeit. physiol. Chem.*, 42: 189 (1904).

Kleinzeller, A.: Fat Formation in *Torulopsis lipofera*, *Biochem. Jour.*, 38: 480–492 (1944).

Lindner, P., und Unger: *Wochschr. Brau.*, 36: 188 (1919).

——: Das Problem der biologischen Fettbildung und Fettgewinnung, *Zeit. angew. Chem.*, 35: 110 (1922).

MacLeod, L. D., and I. Smedley-MacLean: Yeast Metabolism, *Biochem. Jour.*, 32: 1571 (1938).

Nadson, G. A., and H. G. Konodkotine: "Fat Yeast," *Endomyces vernalis*, as a Source of Fat for Foods and Technical Purposes, *Wochschr. Brau.*, 41: 249 (1924), *Chem. Abstracts*, 19: 2219 (1925).

Nageli, C., und O. Loew: Über die chemische Zusammensetzung der Hefe, *Ann.*, 193: 322 (1878).

Rippel, A.: Biologische Fettgewinnung durch Hefen im Luftungsverfahren, *Naturwissenschaften*, 31: 248 (1943).

Smedley-MacLean, I.: The Conditions Influencing the Formation of Fat by the Yeast Cell, *Biochem. Jour.*, 16: 370 (1922).

——. The Biochemical Synthesis of Fat from Carbohydrate, *Ergeb. Enzymforsch.*, 5: 285 (1936).

—— and D. Hoffert: The Carbohydrate and Fat Metabolism of Yeast, *Biochem. Jour.*, 17: 720 (1923).

—— and ——. The Carbohydrate and Fat Metabolism of Yeast, II. The Influence of Phosphates on the Storage of Fat and Carbohydrate in the Cell, *Biochem. Jour.*, 18: 1273 (1924).

—— and ——: The Carbohydrate and Fat Metabolism of Yeast, III. Nature of the Intermediate Stages. *Biochem. Jour.*, 20: 343 (1926).

—— and E. M. Thomas: The Nature of Yeast Fat, *Dept. Sci. Ind. Research (Brit.) Food Investigation, Mem. 9*, 1920.

Starkey, R. L.: Lipid Production by a Soil Yeast, *Jour. Bact.*, 51: 33–50 (1946).

Wieland, H., und F. Wille: Weitere Versuche über die Dehydrierung von Alkohol durch Hefe. Über den Mechanismus der Oxydationsvorgänge, XLI, *Ann.*, 515: 260 (1935).

Some References on Riboflavin

Burkholder, P. R.: Synthesis of Riboflavin by a Yeast, *Proc. Nat. Acad. Sci.*, 29: 166–172 (1943).

——: Influence of Some Environmental Factors upon the Production of Riboflavin by Yeast, *Arch. Biochem.*, 3: 121–129 (1943).

—— Fermentation Process for the Production of Riboflavin (Vitamin B₂), U.S. Patent 2,363,227, Nov. 21, 1914.

Dale, J. K.: Process for Obtaining Crystalline Riboflavin, U.S. Patent 2,421,142, May 27, 1917.

Guilliermond, A.: Sur un Champignon nouveau, parasite des capsules du Cotonnier, l'Eremothecium Ashbyii et ses relations possibles avec le Spermophthora Gossypii et les Ascomycetes, *Compt. rend. acad. sci., Paris* 200: 1556–1558 (1935)

————, M. FONTAINE, et A. RAFFY: Sur l'existence dans l'Eremothecium Ashbyii d'un pigment jaune se rapportant au groupe des flavines, *Compt. rend. acad. sci. Paris.*, 201: 1077–1080 (1935).

HINES, G. E., Jr.: Process for Recovery of Riboflavin, U.S. Patent 2,367,644, Jan. 16, 1945.

————: Process for the Recovery of Riboflavin, U.S. Patent, 2,387,023, Oct. 16, 1945.

KERESZTESY, J. C.: Production of Riboflavin from Fermentation Residues, U.S. Patent 2,355,220, Aug. 8, 1944

McMILLAN, G. E.: Process for Recovering Crystalline Riboflavin, U.S. Patent 2,367,646, Jan. 16, 1945

MIRIMANOFF, A. et A. RAFFY. Obtention de la flavine à l'état cristallisé à partir d'Eremothecium Ashbyii, *Compt. rend. acad. sci.*, 206: 1507–1509 (1938).

———— et ————: Obtention de flavine à partir d'un ascomycète., *Eremothecium Ashbyii, "Helv. Chim. Acta*, 21: 1001–1006" (1938).

PHERSUA, H. D.: Production of Riboflavin, U.S. Patent 2,400,710, May 21, 1946.

ROGOSA, M.: Synthesis of Riboflavin by Lactose-fermenting Yeasts, *Jour. Bact.,* 45 (No. 5): 459–460 (1943).

RUDERT, P. J.: Production of Riboflavin by Biochemical Methods, U.S. Patent 2,374,503, Apr. 24, 1945

SCHOPFER, W. H. : La biotine, l'aneurine et le méso-inositol, facteurs de croissance pour *Eremothecium Ashbyii* Guillermond La biosynthèse de la riboflavine, *Helv. Chim. Acta*, 27: 1017–1032 (1944)

TANNER, F. W., JR., C. VOJNOVICH, and J. M. VAN LANEN: Riboflavin Production by *Candida Species, Science*, 101 (No 2619): 180–181 (February, 1945).

WICKERHAM, L. J., M. H. FLICKINGER, and R. M. JOHNSTON: The Production of Riboflavin by *Ashbya gossypii, Arch. Biochem.*, 9 (No. 1): 95–98 (1946).

CHAPTER X

THE GLYCEROL FERMENTATION

Glycerol $\begin{pmatrix} CH_2OH \\ | \\ CHOH \\ | \\ CH_2OH \end{pmatrix}$ is the simplest alcohol that contains three

hydroxyl groups. It is a chemical substance that has many uses in industry and in the arts

In industry, glycerol is prepared principally by the saponification of fats and oils in the making of soaps. Synthetic glycerol may be made from propylene or propane A very small amount of free glycerol is found in palm and in some less common oils. During World War I, approximately 1,000 tons of glycerol per month were manufactured by a fermentation method, known as the "sulphite process"[1]

Historical.—In his studies of wines and beers, Pasteur found that glycerol was formed regularly by yeasts to the extent of 2 5 to 3 6 per cent on the basis of the weight of the sugar fermented

About three years before the beginning of World War I, Neuberg and his fellow workers commenced to publish some of the results of their studies concerned with the mechanism of the ethyl alcohol fermentation by yeasts Neuberg's scheme for the course of events during the normal fermentation is shown in Chap. V. While experimenting on aldehyde fixation with sodium sulphite in an attempt to elucidate further facts concerning the mechanism of the fermentation, he discovered that a large increase occurred in the amount of glycerol formed when sulphite was added to the fermentation medium He suggested the following reaction

$$C_6H_{12}O_6 + Na_2SO_3 + H_2O \rightarrow NaHCO_3 + CH_3 CHO\ NaHSO_3 + C_3H_8O_3$$

From the preceding equation, 100 g of hexose theoretically yields 51 g of glycerol when 70 g of anhydrous sodium sulphite is used At the same time, slightly more than 24 4 g of acetaldehyde is fixed The ratio of acetaldehyde to glycerol is 24 4 51, or 1 2 09

[1] LAWRIE, J W, "Glycerol and Glycols," Reinhold Publishing Corporation, New York, 1928

Table 67 shows the yields of glycerol and aldehyde obtained when the proportions of sodium sulphite were varied:

TABLE 67.—EFFECT OF VARIOUS AMOUNTS OF SODIUM SULPHITE ON YIELDS OF GLYCEROL AND ALDEHYDE[1]

Na$_2$SO$_3$, parts used	Sugar, parts used	Aldehyde, parts produced	Glycerol, parts produced	Ratio of aldehyde to glycerol
33	100	11.90	23.37	1:1.96
50	100	12.52	24.86	1:1.98
75	100	13.89	27.62	1:1.98
150	100	18.65	36.90	1:1.98

[1] LAWRIE, J. W., "Glycerol and Glycols," Reinhold Publishing Corporation, New York, 1928

An examination of this table indicates that the ratio of aldehyde to glycerol that was obtained was fairly close to the theoretical and independent of the quantity of sulphite used.

Neuberg's second and third schemes indicate the courses of the fermentations when a sulphite and an alkaline medium are used.

Methods.—Increased yields of glycerol may be secured by the use of acids; by acid salts; by neutral salts; by alkalies; or by alkaline salts

Three well-known processes for the production of glycerol by fermentation are the sulphite process, with which the names of Connstein and Ludecke are associated and which was used on a large scale by Germany during World War I; the Cocking and Lilly process in which a mixture of sulphite and bisulphite is used; and the alkaline process developed by Eoff in the United States.

In all these processes the basic medium contains a fermentable sugar and, if necessary, added nutrient salts. The medium is inoculated with a yeast culture and maintained at the optimum temperature for the fermentation, usually 30 to 37°C. From time to time during the fermentation, small portions of the agents that cause increased yields of glycerol are added to the mash. The normal course of the fermentation is altered.

The Sulphite Process.—The basis for this process is the fixation of acetaldehyde by sodium sulphite:

$$CH_3 CHO + Na_2SO_3 + H_2O \rightarrow CH_3 CHO \cdot HSO_3Na + NaOH$$
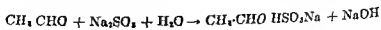
Normally acetaldehyde is reduced in large part to ethyl alcohol during the fermentation of sugars by yeasts. But when this hydrogen acceptor becomes fixed by sulphites, a second molecule in the mash, a triose pro-

duced from hexose, acts as the main hydrogen acceptor and becomes reduced to glycerol (see page 151 of Chap. V for the reactions).

The effect of various concentrations of sodium sulphite on the glycerol yield is shown in the following table:

TABLE 68.—EFFECT OF VARIOUS CONCENTRATIONS OF SODIUM SULPHITE ON YIELDS OF GLYCEROL[1]

Sodium sulphite, parts by weight	Sugar, parts by weight	Glycerol yield, based on sugar
40	100	23 1
67	100	24 8
80	100	27 3
100	100	30 1
120	100	33 0
150	100	34 6
200	100	36 7

[1] LAWRIE, J. W., "Glycerol and Glycols," Reinhold Publishing Corporation, New York, 1928.

Increasing the amounts of sodium sulphite caused a corresponding increase in the quantity of fixed aldehyde and decreases in the yields of alcohol and carbon dioxide.

Salts giving an alkaline reaction were found to increase the yield of glycerol, but alkaline media favored the growth of contaminants. By using large amounts of sodium sulphite, the danger of infection was eliminated.

The sulphite process that the Germans used during World War I was founded on the following example:[1] Sucrose (1 kg.), nutrient salts (50 g. of ammonium nitrate and 7.5 g. of dipotassium phosphate), and sodium sulphite (400 g.) were dissolved in water (10 liters) and inoculated with fresh yeast (100 g), the mash being permitted to incubate at 30°C. for 2 to 2.5 days.

Although beet sugar was used by the Germans in the sulphite process, it has been stated that "neither the kind of sugar nor the variety of yeast influence the fermentation."[2] Yeast may be used repeatedly, if purified between fermentations.

It is important to control the temperature of the fermentation, especially during the summer months.

Glycerol may be recovered by the following method of Connstein and Ludecke[2]: Alcohol and acetaldehyde are separated by distillation. The sulphite in the spent slop is precipitated as calcium sulphite by the addi-

[1] MAY, O L., and H T. HERRICK, *Ind. Eng Chem*, 22: 1172 (1930)
[2] LAWRIE, *op cit.*

tion of calcium oxide, hydroxide, or chloride, and filtered out   Calcium salts that remain in the filtrate are treated with sodium carbonate to form the insoluble calcium carbonate, which is removed.   Technically pure glycerol is obtained by distilling the liquor, which contains glycerol and sodium chloride mainly, under reduced pressure.

In order to determine the amount of glycerol present in the mash, the filtrate from which the carbonates have been removed may be reduced to a sirup by evaporation and then extracted with ethyl alcohol.   Glycerol and alcohol are separated by the process of evaporation.   The glycerol may then be determined by the Zeisel-Fant isopropyl iodine method or distilled and weighed as such.

For an extended discussion of the recovery and determination of glycerol, in which there are certain problems, the interested reader is referred to "Glycerol and Glycols," by Dr. J. W. Lawrie.

Theoretically, a yield of 51 parts of glycerol should be obtained from 100 parts of hexose.   Yields of this magnitude are not obtained by the sulphite process, but considerable of the glycerol is lost owing to low efficiency in the recovery process.   On the basis of the weight of sucrose fermented, approximately 20 to 25 per cent of glycerol, 30 per cent of alcohol, and 5 per cent of acetaldehyde were obtained by Connstein and Ludecke   Actually it required usually 10 to 12 kg. of refined sugar to produce 1 kg. of dynamite glycerol by this process on an industrial basis.

The Cocking-Lilly Process.—This process is a modification of the sulphite process of Connstein and Ludecke.   Mixtures of normal sulphites and bisulphites of the alkali metals are added to the fermenting mash   The fermentation time is much shorter than in the normal sulphite process.   Yields should be higher.

The addition of bisulphites to a fermenting mash causes the acetaldehyde to be fixed at an earlier stage in the fermentation than is usually possible and aids in neutralizing the bicarbonate formed.

Bisulphites are antiseptic in nature   Owing to their antiseptic properties, they cannot be used alone in the fermentation mash in large amounts.   But they may be mixed with normal sulphites to produce a combination which is neutral to litmus and which does not demonstrate too strongly inhibitory action toward the yeast.

A bisulphite is formed when sodium sulphite is added to a fermenting mash, but it reacts with acetaldehyde to form the fixation product:

$$Na_2SO_3 + H_2O + CO_2 \rightarrow NaHSO_3 + NaHCO_3$$

When a mixture of sulphites (molecule for molecule) is added to fermenting media, the reaction is as follows:

$$Na_2SO_3 + NaHSO_3 + CO_2 + H_2O \rightarrow 2NaHSO_3 + NaHCO_3$$

During the fermentation, an aqueous solution of a bisulphite (preferably sodium) is added in successive small portions, or aqueous solutions of a mixture of sulphites, containing increasing proportions of bisulphite, are added as the fermentation proceeds. The relative proportions of the sulphites should be such that the combination is neutral or approximately neutral to litmus.

The total quantity of sulphites to be added to a mash may be calculated either on the basis of the yield of glycerol desired or on the basis of the theoretical yield of glycerol.

The Eoff Process.—This process, one for producing glycerol in an alkaline medium, was developed by Eoff, Linder, and Beyer[1] of the Division of Chemistry of the Bureau of Internal Revenue Investigations were initiated in 1917 as a result of reports that glycerol was being made by fermentation methods in Germany.

The Process in Brief.—A nutrient solution containing a sugar is inoculated with a selected "trained" yeast and incubated at 30 to 32°C. An alkaline reacting compound is added to the fermenting medium in amounts up to approximately 5 per cent, in accordance with a definite schedule The fermentation proceeds usually from 5 to 7 days before the glycerol is recovered.

Details of the Process—Blackstrap molasses, solutions containing mixtures of corn sugar and malt sprouts, and solutions of sucrose containing nutrient salts have been used successfully as media. The optimum concentration of sugar is 17 5 to 20 g per 100 cc. Ammonium chloride in small amounts aids in the production of glycerol.

Two yeasts were found to produce the highest yields of glycerol: *Saccharomyces ellipsoideus* (var. Steinberg) and *S. ellipsoideus* (var California wine yeast), the former yeast giving the best results.

Yeast that has been "trained" or acclimated to growth in an alkaline medium produces the highest yields of glycerol The main mash is inoculated with a starter that represents approximately 10 per cent of its volume The first culture of yeast may be grown in a small flask. Training is given to the yeast by adding 0 5 to 1 per cent of sodium carbonate (calculated on the basis of the weight of the solution) to the culture The first effect is to stop fermentation. This is transitory When the fermentation becomes active again, a larger volume of mash is inoculated with 5 to 10 per cent of trained yeast. Alkali is added to this mash, and when the culture becomes active a proportionally larger mash is inoculated. This process is repeated until the starter eventually

[1] Eoff, J R , W V Linder, and G F Beyer, *Ind. Eng. Chem.*, **11**: 842 (1919)

tion of calcium oxide, hydroxide, or chloride, and filtered out. Calcium
salts that remain in the filtrate are treated with sodium carbonate to
form the insoluble calcium carbonate, which is removed. Technically
pure glycerol is obtained by distilling the liquor, which contains glycerol
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properties, they cannot be used alone in the fermentation mash in large
amounts   But they may be mixed with normal sulphites to produce a
combination which is neutral to litmus and which does not demonstrate
too strongly inhibitory action toward the yeast.

A bisulphite is formed when sodium sulphite is added to a fermenting
mash, but it reacts with acetaldehyde to form the fixation product:

$$Na_2SO_3 + H_2O + CO_2 \rightarrow NaHSO_3 + NaHCO_3$$

When a mixture of sulphites (molecule for molecule) is added to
fermenting media, the reaction is as follows:

$$Na_2SO_3 + NaHSO_3 + CO_2 + H_2O \rightarrow 2NaHSO_3 + NaHCO_3$$

During the fermentation, an aqueous solution of a bisulphite (preferably sodium) is added in successive small portions, or aqueous solutions of a mixture of sulphites, containing increasing proportions of bisulphite, are added as the fermentation proceeds. The relative proportions of the sulphites should be such that the combination is neutral or approximately neutral to litmus.

The total quantity of sulphites to be added to a mash may be calculated either on the basis of the yield of glycerol desired or on the basis of the theoretical yield of glycerol.

The Eoff Process.—This process, one for producing glycerol in an alkaline medium, was developed by Eoff, Linder, and Beyer[1] of the Division of Chemistry of the Bureau of Internal Revenue. Investigations were initiated in 1917 as a result of reports that glycerol was being made by fermentation methods in Germany.

The Process in Brief.—A nutrient solution containing a sugar is inoculated with a selected "trained" yeast and incubated at 30 to 32°C. An alkaline reacting compound is added to the fermenting medium in amounts up to approximately 5 per cent, in accordance with a definite schedule. The fermentation proceeds usually from 5 to 7 days before the glycerol is recovered.

Details of the Process—Blackstrap molasses, solutions containing mixtures of corn sugar and malt sprouts, and solutions of sucrose containing nutrient salts have been used successfully as media. The optimum concentration of sugar is 17.5 to 20 g per 100 cc. Ammonium chloride in small amounts aids in the production of glycerol.

Two yeasts were found to produce the highest yields of glycerol *Saccharomyces ellipsoideus* (var. Steinberg) and *S. ellipsoideus* (var California wine yeast), the former yeast giving the best results.

Yeast that has been "trained" or acclimated to growth in an alkaline medium produces the highest yields of glycerol. The main mash is inoculated with a starter that represents approximately 10 per cent of its volume. The first culture of yeast may be grown in a small flask. Training is given to the yeast by adding 0.5 to 1 per cent of sodium carbonate (calculated on the basis of the weight of the solution) to the culture. The first effect is to stop fermentation. This is transitory. When the fermentation becomes active again, a larger volume of mash is inoculated with 5 to 10 per cent of trained yeast. Alkali is added to this mash, and when the culture becomes active a proportionally larger mash is inoculated. This process is repeated until the starter eventually

[1] EOFF, J. R., W. V. LINDER, and G. F. BEYER, *Ind Eng. Chem*, 11:842 (1919)

secured is sufficiently large to seed the main mash. For further details, the reader is referred to the report submitted by Eoff, Linder, and Beyer to the Commissioner of Internal Revenue on May 6, 1918. The facts contained in this report were reproduced in the *Journal of Industrial and Engineering Chemistry*, **11**: 842 (1919).

Sodium carbonate in the form of soda ash is preferable for use in the fermentation on account of its comparatively low cost, although potassium carbonate produces results that are just as favorable. Sodium and potassium hydroxides, sodium perborate, and other substances may be used also to produce alkalinity. Not much more than 5 per cent of sodium carbonate (calculated on the weight of the mash) should be used, for otherwise the fermentation will be permanently inhibited. On the other hand, the highest possible concentration (close to 5 per cent) of the carbonate should be employed, for the yield of glycerol is increased by increasing the alkalinity of the mash almost to the limit of endurance of the yeast. The final amount of alkali in 100 cc. of mash may be equivalent to 95 cc. of normal sodium hydroxide.

As soon as the fermentation becomes vigorous, sodium carbonate is added as a solid. A precipitate forms after the addition of the carbonate, and the evolution of gas temporarily ceases. The precipitate disappears gradually, and fermentation again proceeds. Thereafter sodium carbonate is added in as large amounts and as rapidly as the yeast will tolerate. Usually the alkali is added in accordance with a definite schedule. Eoff was in favor of adding it in 5 portions: the first portion containing approximately 12.5 per cent of the total carbonate to be added, the second portion, about 22 per cent; the third, about 31 per cent; the fourth, about 22 per cent; and the fifth, about 12.5 per cent

During the fermentation the temperature should be kept within the limits of 30 to 32°C. in order to ensure high yields.

From 20 to 25 per cent of the sugar of the mash is converted to glycerol, while considerable quantities of ethyl alcohol and carbon dioxide are produced

Eoff and his associates obtained successful results from the fermentation of mashes of 2,000-gal. capacity.

The I.   and Hickey[1]

in which ammonium sulphite and/or ammonium bisulphite are present in excess   The pH is maintained at about 6.8 by means of a pH recorder-controller, using sulphur dioxide or sulphurous acid.

[1] FULMER, E. I, L. A. UNDERKOFLER, and R J. HICKEY, U S. Patent 2,416,745, Mar. 4, 1947.

The process is carried out as follows A medium, of which the one described below is typical, is prepared

Ingredient	Weight per Liter
Sugar (as dextrose)	150 g
Corn steep liquor (heavy)	2 0–4 5 g
NH₄Cl	1.5 g.
K₂HPO₄ 3H₂O	0 75 g.
KH₂PO₄	0 75 g.
MgSO₄·7H₂O	0 2 g
CaCl₂	0 1 g

Ammonium sulphite and/or ammonium bisulphite is used in an amount equivalent to 60 g. of SO₂. The pH of the medium is adjusted to 6.5 The medium is then inoculated with a culture of yeast acclimatized to ammonium sulphite. An amount of inoculum representing 10 per cent of the volume of the medium is satisfactory. After the fermentation becomes active, a portion of the ammonium sulphite and/or bisulphite is added; a 5-g portion may be added, at intervals of approximately 6 hr. The sulphite may be added as a solid or as ammonia or ammonium hydroxide combined with sulphur dioxide or sulphurous acid.

In recovering the glycerol, there is added a quantity of calcium hydroxide in excess of that required to make the medium alkaline The volatile substances are removed by distillation, and the ammonia, acetaldehyde, and ethanol are separated The solids may be removed by centrifuging or filtering, after which carbon dioxide is added to precipitate the calcium in solution. The precipitate of calcium carbonate is removed by centrifuging or filtering. Glycerol remains in the residue

In an earlier patent, Fulmer and his associates[1] described a process for producing glycerol in an acid medium containing magnesium sulphite as the aldehyde-fixing agent The pH was maintained at 6.5 with a pH recorder-controller, by using 50 per cent acetic acid. The fermentation was complete in 96 hr Yields were obtained of 23 15 per cent of glycerol, *based on the dextrose used.*

The Schade-Färber Process.—Schade and Färber,[2] and Schade[3] have patented processes for producing glycerol in which the volatile constituents are removed from the fermentation medium by bubbling nitrogen, oxygen, or air through it. The principal volatile substances are ethanol, carbon dioxide, and acetaldehyde They may be recovered by passage through special scrubbing towers.

[1] FULMER, E. I, L A. UNDERKOFLER, and R J HICKEY, U S Patent 2,388,810. Nov 13, 1945
[2] SCHADE, A L, and E FÄRBER, U S Patent 2,414,828, Jan 28, 1947
[3] SCHADE, A L, U S Patent 2,428,766, Oct 7, 1947

In one process, glycerol is produced in the presence of magnesium carbonate. The pH is maintained between 7 and 8 by the addition of 10 to 20 parts of magnesium carbonate for each 100 parts of fermentable carbohydrate present initially in the medium. For example, 10 liters of medium (obtained by hydrolyzing wheat) containing 1,700 g. of reducing sugar were added, together with 170 g. of compressed yeast (of 72 per cent moisture content), to a fermentation vat that was equipped with a stirrer, a gas disperser, and gas outlets. The vat was located in a room maintained at a constant temperature of 32°C. The medium was aerated at the rate of 1 liter per min. The pH was kept in the range 7.9 to 7.2 by the addition of a total of 170 g. of magnesium carbonate. Within 26 hr., almost all of the fermentable sugar had been consumed. A yield was obtained of 310 g. of pure glycerol and 400 g. of yeast, which could be reused.

In a second process, the volatile products are removed as they are produced and the pH is maintained between 6.9 and 7.3 by the addition of neutralizing agents, such as hydroxides, alkaline-reacting phosphates, carbonates of the alkali metals, etc. It is claimed that media containing 10 to 20 per cent of carbohydrates may be fermented in 12 to 24 hr. at 34 to 37°C. In one example, 10 liters of a solution containing 1,000 g. of reducing sugars were placed with 100 g. of compressed yeast of 72 per cent moisture content in a vat equipped and aerated as described above. The pH was maintained at 6.9 to 7.3 by the addition of a 5 per cent solution of sodium hydroxide. Within 12 hr., 795 g. of the fermentable sugar had been consumed. There were recovered 310 g. of yeast (72 per cent moisture) and 150 g. of pure glycerol.

Glycerol from Molasses.—Duchenne[1] revealed that glycerol was being produced from molasses on an industrial scale in South Africa, using an alkaline fermentation. He described the production of glycerol, using calcium sulphite, and reported that a sugar factory that produced 8,000 tons of molasses a year could manufacture 6 tons of glycerol and 1,000 gal. of ethanol each day.

Glycerol from Hydrolyzed Starch-containing Materials.—Lees[2] studied the production of glycerol from starch-containing materials, using sodium or magnesium sulphite. Enzyme-saccharified starch-containing materials were unsatisfactory for glycerol production, since maltose was the chief end-product and maltose, even in pure form, was fermented very slowly. Acid-hydrolyzed corn starch or dry-milled corn products were satisfactory for glycerol production. With magnesium sulphite, yields of

[1] Duchenne, J. O., *Proc. 16th Ann. Congress S. African Sugar Technol. Assoc.*, pp. 45–47 (Apr. 21–23, 1942).

[2] Lees, T. M., *Iowa State Coll., Jour. Science*, 19 (No. 1) 38 (1944).

22 to 24 per cent glycerol (on the basis of dextrose) were obtained; with sodium sulphite, yields as high as 30 per cent were secured. However, it was necessary to use large inocula of yeast and high concentrations of sodium sulphite to obtain yields above 25 per cent.

Schade and Farber[1] and Schade[1] used hydrolyzed wheat and other grains successfully for glycerol production.

Theoretical.—According to Neuberg's scheme for the production of glycerol in an alkaline medium, 1 molecule of acetic acid is produced with each 2 molecules of glycerol:

$$2C_6H_{12}O_6 + H_2O \longrightarrow CH_3COOH + 2C_3H_8O_3 + C_2H_5OH + 2CO_2$$

The ratio of glycerol to acetic acid is 184·60, or 3.07:1. Some actual ratios varied from 2.91.1 to 3 12 1.[2]

Glycerol from Synthetic Sugar.—Ludecke[3] has patented a process for producing glycerol from the sugar or sugar sirup derived from the condensation of formaldehyde in an alkaline solution. (For details of the condensation process, consult German Patent 590236)

Uses of Glycerol.—Glycerol finds a wide variety of uses. These include its use as a solvent; as a sweetening agent; as a constituent of ointments, lotions, antiseptics, adhesives, and inks; as a food; and as an antifreeze agent. It is used in the preparation of biological media and nitroglycerine[4] It may be used in the manufacture of synthetic rubber, glycerine-litharge cements, and modeling clays.[5] New uses for this trihydric alcohol are continually being found[6]

Further Information.—Additional information concerning the glycerol fermentation may be obtained by a study of the publications listed at the end of this chapter. Much valuable data will be found in the patent literature, especially that of Germany. Some patents have not been published in the latter country owing to their secret nature

References on Glycerol Fermentation and Glycerol

Duchenne, J O . Manufacture of Glycerin from Sugar by Fermentation, *Proc 16th Ann Congress S African Sugar Technol Assoc*, pp 45–47 (Apr 21–23, 1942).

Eoff, J R , W V Linder, and G F Beyer Report to the Commissioner of Internal Revenue, U S Dept of the Treasury, May 6, 1918

———, ———, and ———: Production of Glycerin from Sugar by Fermentation, *Ind Eng Chem*, 11: 842 (1919)

Gillaudeu, A · Glycerol-Liberation, Recovery, and Refining, *Ind Eng Chem*, 29: 729 (1937).

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[1] *Loc cit*

[2] *Lawrie, op cit*

[3] Lüdecke, K , German Patent 658017, Apr 27, 1938

[4] Lessig, M A , and J R Murphy, Glycerine, *1m Professional Pharmacist*, June July, August, 1936

[5] "Glycerine Facts," Glycerine Producers' Association, New York, 1949

Henneberg, W.: "Handbuch der Gärungsbakteriologie," 2d ed , Paul Parey, Berlin, 1926.

Lawrie, J. W.: "Glycerol and Glycols," Reinhold Publishing Corporation, Inc, New York, 1928.

Leffingwell, G : Glycerine Facts, *Glycerine Producers' Assoc.*, Trade Paper Bull, New York, 1938, 1939, 1940.

Lees, T M.: The Fermentative Production of Glycerol, *Iowa State Coll. Jour. Sa*, 19 (No. 1): 38–40 (1941)

Lessler, M A, and J R Murphy· Glycerine—Its Role in Medicine, *Am Profes Pharmacist*, June, July, August, 1936

May, O. E, and H T Herrick: Some Minor Industrial Fermentations, *Ind Eng Chem.*, 22 : 1172 (1930)

Stephenson, M "Bacterial Metabolism," 3d ed., Longmans, Green & Company, New York, 1948

Some Patents

Aktieselkabet Dansk Gaerings-Industri, Danish Patent 62,582, Aug. 21, 1944.

Carothers, W H, J W Hill, and F. J. L. Van Natta: Method of Manufacturing Fermentation Glycerol, U S. Patent 1,936,497, Nov. 21, 1933

Cocking, A T, and C H. Lilly: Production of Glycerine by Fermentation, US Patent 1,425,838, Aug. 15, 1922. Also British Patent 164,034, 1921.

Connstein, W, and K. Lüdecke: Process for the Manufacture of Propantriol from Sugar, U S. Patent 1,511,751, Oct. 14, 1921. Also German Patents 298593 298596, 1915, 13860, 1917, etc.

Loff, J R.: Process of Manufacturing Glycerol, U.S. Patent 1,288,398, Dec. 17, 1918

Fulmer, E. I, L A. Underkofler, and R. J. Hickey: Glycerol Production, US Patent 2,338,810, Nov. 13, 1915.

————, ————, and ————: Fermentative Production of Glycerol, US Patent 2,416,745, Mar 4, 1947.

Hamlin, H · British Patent 488,464, July 7, 1938; also French Patent 829,263, June 17, 1938

Henkel & Cie G m b H.. German Patent 664576, Aug. 29, 1938

Hildebrandt, F M, and N M Enn: Glycerol Fermentation Process, U.S Patent 2,169,215, Aug 15, 1939

Lawrie, J M : Acetic Acid Manufacture from Glycerol Fermentation Residues, U S Patent 1,909,183, May 16, 1933.

Lilly, C H · Manufacture of Glycerine, U.S. Patent 1,987,260, Jan. 8, 1935

Lüdecke, K : Verfahren zur Gewinnung von Glycerin aus kohlehydraten durch Gärung, German Patent 658017, published Apr. 27, 1938

Neish, A C., G. A Ledingham, and A. C. Blackwood: Production of Glycerol b Fermentation, U S Patent 2,432,032, Dec. 2, 1947

Norddeutsche Hefemdustrie A -G. German Patent 664575, Aug 29, 1938

Schade, A L · Production of Glycerol and Yeast by Fermentation, U S Patent 2,428,766, Oct 7, 1947

————, and E Farber: Process for the Manufacturing of Glycerin, U S Paten 2,414,838, Jan 28, 1947

Walmsley, R A : Recovery of Glycerin from Fermented Liquors, U S Patent 2,422,453, June 17, 1947

Werner, K : Process for the Recovery of Glycerine, U.S. Patent 2,153,704, Apr 11 1939

CHAPTER XI

THE BACTERIA

Bacteria, like molds, because of their great range of types and the variety of their action, may be very useful, extremely harmful, or, so far as present knowledge goes, of no particular significance to man It is well known that bacteria cause diseases in animals and in plants, decomposition of foodstuffs, and are responsible for many biochemical changes in nature. Some of the useful applications of bacteria are considered in the chapters that follow

The bacteria that are used in industry may be divided by their relation to free oxygen into two main groups· the anaerobic bacteria and the aerobic bacteria The former group, which may be considered to include also the microaerophiles, includes the largest number of useful types of bacteria from the industrial standpoint Bacteria active in producing butanol, acetone, ethanol, isopropanol, lactic acid, fermented milk products, cheese, and other substances of recognized or potential value are included in this group, although not all the organisms concerned in the production of some of these compounds are anaerobic in character. The outstanding aerobic bacteria include the well-known *Acetobacter* group of bacteria, some of which have the ability to produce acetic acid, gluconic acid, dihydroxyacetone, sorbose, and other substances from suitable substrates by oxidative means. Among aerobes, also, may be placed the organisms concerned in the acetone-ethanol fermentation

Table 69 gives data concerning some important fermentation bacteria, the fermentations with which they are associated, their oxygen relationships, and the optimum temperatures for their growth and biochemical activity

Table 70 lists some of the products of bacterial fermentation, some of the materials from which these are formed, and the general class of the bacteria concerned.

TABLE 69.—IMPORTANT FERMENTATION BACTERIA: FERMENTATIONS, RELATION TO OXYGEN, AND OPTIMUM TEMPERATURES FOR GROWTH

Family	Genus and species	Fermentations with which commonly associated	Relationship to air[1]	Optimum temperature, °C.[1]
Acetobacteriaceae Bergey, Breed, and Murray (*Pseudomonadaceae* in latest ed of Bergey's Manual)	*Acetobacter kashigaki*	Gluconic acid	Aerobic	30-35
	A suboxydans	Dihydroxyacetone, sorbose, acetic acid, gluconic acid, etc.	Aerobic	30
	A xylinum	Sorbose, acetic acid, etc.	Aerobic	28
	Bacterium curvum	Vinegar (quick process)	Aerobic	25-30
	Bact. orleanense	Vinegar (Orleans and quick processes)	Aerobic	20-30
	Bact schutzenbachii	Vinegar (quick process)	Aerobic	25-27.5
Lactobacteriaceae Orla-Jensen				
Tribe Streptococceae	*Leuconostoc mesenteroides*	Sauerkraut	Facultative aerobic	21-25
	Streptococcus lactis	Lactic acid	Facultative aerobic	
Tribe Lactobacilleae	*Lactobacillus acidophilus*	Acidophilus milk	Microaerophilic	37
	Lactobacillus bulgaricus	Milk products, lactic acid	Aerobic to anaerobic	45-30
	L breve	Milk products, sauerkraut, ensilage, etc		30
	L casei	Lactic acid	Microaerophilic	30
	L. delbrueckii	Lactic acid	Microaerophilic	45
	L leichmannii	Lactic acid	Microaerophilic	36
	L plantarum	Sauerkraut, pickles, milk products, etc.	Microaerophilic	30
	Propionibacterium freudenreichii	Swiss cheese, propionic acid	Anaerobic	
	P shermanii, etc.	Propionic acid, etc.	Anaerobic	
Bacillaceae Fischer Genus I	*Bacillus macerans* Schardinger	Ethanol-acetone	Aerobic	42-45
	B acetoethylicus Northrop et al. (probably identical with *B. macerans*)	Ethanol-acetone	Aerobic	40-43
Genus II	*Clostridium acetobutylicum* McCoy, Fred, Peterson, & Hastings	Butanol-acetone	Anaerobic	37
	Cl. butyricum Prazmowski	Butanol-acetone	Anaerobic	30-37
	Cl felsineum	Retting, butanol-acetone	Anaerobic	37

[1] Data secured from "Bergey's Manual of Determinative Bacteriology," 6th ed, The Williams & Wilkins Company, Baltimore, 1948

TABLE 70.—PRODUCTS OF BACTERIAL FERMENTATION

Fermentation product	Materials from which formed	Organisms causing change
Acetaldehyde	Sugars, ethanol, pyruvic acid, etc.	Acetic acid and butanol-acetone bacteria, Escherichia coli, etc.
Acetic acid	Ethanol, glucose, cellulose, etc	Acetic acid, heterofermentative lactic acid, butanol-acetone, isopropanol, propionic acid, and cellulose-fermenting bacteria
Acetoacetic acid	Acetic acid	Butanol-acetone bacteria
Acetone	Starch, sugars, acetoacetic acid, acetic acid, isopropanol	Butanol-acetone, ethanol-acetone, isopropanol, and acetic acid bacteria
Acetylmethylcarbinol	2 3-butyleneglycol, sugars, pyruvic acid, etc	Aerobacter aerogenes, butanol-acetone and acetic acid bacteria
Butanol	Starch, sugars, butyric acid, etc.	Butanol-acetone and isopropanol bacteria
Butyric acid	Starch, sugars, cellulose, acetic acid, etc	Butyric acid and cellulose-fermenting bacteria
2 3-butyleneglycol	Glucose, diacetyl	A. aerogenes
Carbon dioxide	Starch, sugars, etc.	Butanol-acetone, A. aerogenes, E coli, and many other bacteria
Dihydroxyacetone	Glycerol	Acetic acid bacteria
Ethanol	Sugars, starch, cellulose, acetaldehyde, etc.	Termobacterium mobile Lindner, butanol-acetone, ethanol-acetone, heterofermentative lactic acid and cellulose-fermenting bacteria, E. coli, etc.
Formic acid	Glucose, cellulose, etc.	Butyric, cellulose-fermenting bacteria, E. coli, etc.
Fructose	Mannitol	Acetic acid bacteria
Galactonic acid	Galactose	Acetic acid bacteria
Galactose	Dulcitol	Acetic acid bacteria
Gluconic acid	Glucose	Acetic acid bacteria
Glycerol	Glucose, etc.	Heterofermentative lactic acid bacteria
Glycerophosphoric acid	Triosephosphoric acid	Lactic acid bacteria, E coli
Hexosediphosphate	Glucose	Lactic acid bacteria, E. coli
Hydrogen gas	Glucose, lactose etc	Butyric acid bacteria, colon-aerogenes group, and other bacteria
Isopropanol	Starch, sugars	Isopropanol-acetone bacteria
2-Ketogluconic acid	Gluconic acid (glucose)	Acetic acid bacteria
5-Ketogluconic acid	Gluconic acid (glucose)	Acetic acid bacteria
Keto acid	Glucose, fructose, etc	Acetic acid bacteria
Lactic acid	Starch, sucrose, glucose fructose, etc	Lactic acid, propionic acid, and cellulose-fermenting bacteria, E. coli, etc
Mannitol	Fructose	Heterofermentative lactic acid bacteria
Mannonic acid	Mannose	Acetic acid bacteria
Mannose	Mannitol	Acetic acid bacteria
Methylglyoxal	Sugars, hexosediphosphate, etc	Acetic acid, butanol-acetone, lactic acid and propionic acid bacteria; E coli
Phosphoglyceric acid	Sugars, hexosediphosphate	Lactic acid bacteria, E. coli
Propanol	Propionic acid	Butanol-acetone bacteria
Propionic acid	Glucose, propanol, lactic acid, glycerol, pyruvic acid	Propionic acid and acetic acid bacteria
Propionaldehyde	Glucose glycerol	Propionic acid bacteria
Propylene glycol	Rhamnose	Bact rhamnosofermentans
Pyruvic acid	Sugars	Butyric, lactic, and propionic acid bacteria
Sorbose	Sorbitol	Acetic acid bacteria
Succinic acid	Glucose, fructose, lactose, and other sugars	Propionic acid bacteria, E. coli, etc.
d Tartaric acid	Glucose	A suboxydans

CHAPTER XII

THE ACETONE-BUTANOL FERMENTATION

There are a number of closely associated fermentations brought about by bacteria, which differ in respect to the quantity and nature of the end products and the conditions necessary for their successful termination. The most important of these fermentations is that in which the main end products are butanol, acetone, and ethanol (neutral solvents); acetic and butyric acids; and carbon dioxide and hydrogen gases. A second important fermentation gives rise to acetone and ethanol as the chief end products of value. In a third type of fermentation, butanol, isopropyl alcohol, and acetone are produced.

Historical.—Normal butyl alcohol was discovered as a regularly occurring constituent of fusel oil by Wurtz in 1852. Pasteur, however, was the first investigator to show that butyl alcohol was a direct product of fermentation. His findings were based on the results of the butyric fermentation of lactic acid and calcium lactate. In a report announcing his discovery he said:

"M Pasteur . . . croit pouvoir affirmer que l'alcool butylique est un produit ordinaire de la fermentation butyrique "

Fitz published a series of articles on fermentations from 1876 to 1884 [1] Among these, he discussed *Bacillus butylicus*. This organism was a sporeformer and produced butyl alcohol, butyric acid, and small amounts of ethyl alcohol, chiefly, with the two gases, carbon dioxide and hydrogen Glycerin, mannite (mannitol), and sucrose were fermented Sucrose was inverted by an enzyme secreted by the bacillus. Since the organism did not form suitable enzymes for hydrolyzing starch and lactose, these materials were not fermented in their native conditions.

In 1887, Gruber [2] described three strains of organisms under the name of *B. amylobacter* (*Clostridium butyricum*). Each of these strains formed butyric acid and butyl alcohol from carbohydrates.

Botkin [3] isolated an anaerobic spore-forming bacillus, which he

[1] REILLY, J , J HICKINBOTTOM, F. R. HENLEY, and A C THAYSEN, *Biochem Jour*, 14: 229 (1920); E. McCOY, E B. FRED, W H. PETERSON, and E G HASTINGS, *Jour Infectious Diseases*, 39: 457 (1926)

[2] GRUBER, M , *Centr Bakt Parasitenk* , 1: 367 (1887)

[3] BOTKIN, S , *Zeit Hyg Infectionskrankh* , 11: 421 (1892)

described in 1892. This organism produced butyl and ethyl alcohols and did not ferment cellulose. According to Botkin, this organism is quite widely distributed in nature and may be isolated from garden soil, milk, and other sources.

B. orthobutylicus, an anaerobe isolated from a calcium tartrate fermentation, was described by Grimbert[1] in 1893. This organism fermented glycerin, mannite, glucose, invert sugar, sucrose, lactose, maltose, galactose, arabinose, starch, potatoes, dextrin, and inulin, but did not ferment calcium lactate, calcium tartrate, or trehalose. Butanol; a little isobutyl alcohol; butyric, acetic, and, in some cases, formic acids; and carbon dioxide and hydrogen gases were produced during the fermentation. Grimbert differentiated his organism from *B butylicus* of Pasteur, *B. amylobacter* of Van Tieghem, *B. butylicus* of Fitz, and *Bacille amylozyme* of Perdrix. He studied the effect of age, the condition of cultivation, and the duration of the fermentation on the proportion of fermentation products and observed with care the reaction, the concentration of carbohydrate compounds, and the use of calcium carbonate in the control of acidity.

In a report on butyl alcohol bacteria, Beijerinck[2] described species named by him *Granulobacter butylicum,* etc (*Granulobacter* is a term applied to microorganisms that demonstrate a blue color with iodine and show a distinct "swelling of the cell at sporulation.") *Granulobacter* replaced the term *Clostridium.*

Duclaux[3] in his "Sur la nutrition intracellulaire," discussed *Amylobacter butylicus,* an organism isolated from potato. The principal products of the fermentation were butyl alcohol, acetic and butyric acids (and lactic acid, sometimes), and the gases, carbon dioxide and hydrogen, in general. He found that the use of calcium carbonate in media caused the production of acids, while its absence favored the production of alcohols. In the same paper, he discussed also *A. ethylicus,* an organism likewise isolated from potato, which produced ethyl alcohol, acetic and lactic acids, and carbon dioxide and hydrogen gases.

In 1897, Emmerling[4] described a facultative anaerobe that produced butyl alcohol from various carbohydrate substances. The fermentation of 100 g. of glycerin yielded 6.3 g of pure butyl alcohol; that of 100 g. of mannite, 10 5 g. of butyl alcohol. Butyric acid was obtained in all fermentations, and ethyl alcohol when glucose was fermented.

[1] GRIMBERT, M L, *Ann Inst Pasteur,* 7: 353 (1893)

[2] BEIJERINCK, M W, *Verhandel Akad Wetenschappen Amsterdam Afdeel. Natuurkunde,* 2e Sectie, 1, no 10 (1893)

[3] DUCLAUX, E., *Ann Inst Pasteur,* 9: 811 (1895)

[4] EMMERLING, O., *Ber.* 30: 451 (1897).

Grassberger and Schattenfroh[1] in 1902 reported the isolation of a motile butyric acid organism from the soil; they claimed that this organism produced butyl alcohol. No alcohol was secured in some later experiments, however.

Winogradsky[2] reported in 1902 on *Cl. pastorianum*, describing its morphology and its properties as a producer of butyric acid. Butyl alcohol, ethyl alcohol, and acetic and butyric acids were produced from the carbohydrates, sucrose and dextrose.

Acetone was first discovered as a fermentation product by Schardinger in 1905. Schardinger[3] described the morphology and biology of the organism, which was named *B. macerans*. Acetone, ethyl alcohol, and acetic and formic acids were produced in fermentation by this organism. Potatoes, or a potato starch medium, with peptone and calcium carbonate were the best substrates found for producing acetone. Using a temperature of 37°C. and permitting the fermentation to continue for 6 days, Schardinger obtained 6.9 per cent by weight of acetone and 20.59 per cent by weight of ethyl alcohol from potato starch.

Buchner and Meisenheimer,[4] using the *B. butylicus* of Fitz, secured yields of 19.6 and 10.4 g. of *n*-butyl and ethyl alcohols, respectively, from 100 g. of glycerin. From glucose, low yields of these solvents were obtained per 100 g (0.7 and 2.8 g.), but there were large quantities of butyric and acetic acids formed (26 0 and 7.5 g., respectively). Calcium carbonate was used in the medium in both cases.

First Successful Commercial Processes.[5]—The need for a synthetic rubber supplied the impetus that resulted in the first successful commercial process. Rubber was synthesized in 1860, but research was continued along this line for many years because it was believed that the natural supply of rubber would not be sufficient to meet the demand.

The year 1909 was a very active one in synthetic rubber research, especially in England, Germany, and Russia, but after this year the production of plantation rubber was such that attempts to produce rubber synthetically no longer seemed feasible.

During the period of greatest activity, the firm of Strange and Graham, Ltd., carried out the most of the investigation in England. Prof. Perkin and his assistant, Weizmann, of Manchester University, and

[1] Grassberger, R., und A. Schattenfroh, *Arch Hyg.*, 42: 219 (1902).

[2] Winogradsky, S., *Centr. Bakt Parasitenk*, Abt. II, 9: 43, 107 (1902).

[3] Schardinger, F, *Centr Bakt Parasitenk.*, Abt. II, 14: 772 (1905).

[4] Buchner, E., und J Meisenheimer, *Ber.*, 41: 1410 (1908).

[5] Gabriel, C L, *Ind Eng Chem.*, 20: 1063 (1928); Kelly, F. C., "One Thing Leads to Another," Houghton Mifflin Company, Boston, 1936, Arzberger, C. F., W. H. Peterson, and E B Fred, *Jour. Biol. Chem*, 44: 465 (1920).

Prof. Fernbach with his assistant, Schoen, of the Pasteur Institute, were employed by the firm to carry on the research.

Synthetic rubber at this time was obtained through the polymerization of isoprene or butadiene. These compounds were best prepared from isoamyl alcohol and n-butyl alcohol, respectively. Isoamyl alcohol was secured from fusel oil, produced in the ethyl alcohol fermentation, of which it constituted approximately 87 per cent. Butadiene was eventually considered the best material to use, and the Germans made much rubber from it synthetically during World War I.

Fernbach and Weizmann in 1911 discovered bacteria that fermented potato starch, yielding amyl alcohol as one of the end products. Butyl alcohol, ethyl alcohol, and acetone were later found in the same fermentation. Fernbach classed the organism responsible for the fermentation as a "bacillus of the type Fitz."

Weizmann in 1912 left the firm of Strange and Graham, Ltd., and continued independent research on fermentation. He isolated an organism which produced nearly four times as much acetone as the Fernbach organism and which was able to ferment starches than potato starch. To Weizmann's organism, the name *B. granulobacter pectinovorum* was given, but this was later changed to *Cl. acetobutylicum,* Weizmann.

During the years 1913 and 1914, the firm of Strange and Graham, Ltd., established plants at Rainham and King's Lynn for producing solvents by fermentation.

With the advent of World War I, it became necessary to seek a new means of producing acetone for use in the manufacture of cordite, an explosive, and the "dopes" for airplane wings. The government made a contract with Strange and Graham, Ltd., to supply the acetone, but the demands could not be met with the use of potatoes as a raw material. At this time, Weizmann's bacillus came to the attention of the government and an order was issued to replace the organism then being used by his organism. Maize was used as a raw material. Distilleries were taken over in England and Canada, and one was rather unsuccessfully started in India.

After war was declared with Germany, the United States purchased two distilleries at Terre Haute, Ind., and established there the Weizmann process. With the termination of World War I, the plants were closed because acetone was no longer needed in large quantities and butanol never had been much in demand.

It was not long, however, before a demand sprang up for n-butyl alcohol in the manufacture of automobile lacquers, for n-butyl acetate was found to be superior to amyl acetate. An American corporation

was organized to carry on the acetone-butanol fermentation in this country. Basic patent rights to the Weizmann and Ricard processes of a world-wide nature were secured, and a new plant was constructed and operated at Peoria, Ill.

In 1918, Higgins[1] reported the construction by the Hercules Powder Company of a plant for fermentation of kelp on the coast of Southern California The seaweed was gathered into ships, macerated, pumped from the ships to a tank at the factory, diluted with water, and fermented at 90°F. with addition of limestone. Acetate of lime was the chief product, but butyric acid was also produced. Acetone was manufactured from the acetate of lime.

During the year 1919 several papers concerning the acetone and acetone-butanol fermentations were published. Nathan[2] described the manufacture of acetone by the Weizmann process from such raw materials as corn and horse chestnuts. Gill[3] described in detail the acetone fermentation by the Weizmann organism and its technical application In another report he discussed the production of acetone and n-butyl alcohol from horse chestnuts by fermentation.[4] Features of the fermentation of horse chestnuts, such as the long period of fermentation, frothing during fermentation and distillation, and the unhealthy appearance of the bacteria were ascribed to the presence of aesculic acid or aesculin By removing as much of the husk as possible, the fermentation was improved. Yields from horse chestnuts averaged about 18 per cent on the basis of dry meal in comparison to 24 per cent from maize.

Industrial Process.—In the industrial manufacture of acetone and butanol by fermentation it is usually necessary to give the raw material some kind of preliminary treatment. When corn is used as the source of carbohydrate, the germ is removed, and the kernels are ground to a coarse meal, for, although the germ is of no value in the ensuing fermentation, it contains considerable oil of commercial importance. The ground meal is mixed with water to give a concentration of 6 to 8 per cent (see Fig 48.) It is then cooked, with agitation, using a steam pressure of 30 lb for 2 hr The starch is rendered soluble and the mash sterilized during this treatment. The cooked mash is blown aseptically through coolers, which reduce the temperature of the mash to about 37°C., to covered fermenters of large capacity where it is inoculated with starters (see Fig 49) and allowed to ferment for 48 to 72 hr. Figure 50 illustrates the general procedure followed in an industrial process.

[1] Higgins, G. A , Ind Eng. Chem., 10: 858 (1918).

[2] Nathan, F., Jour. Soc Chem. Ind , 38: 271–273T (1919).

[3] Gill, A., Jour. Soc Chem Ind., 38: 273–282T (1919).

[4] Gill, A , op. cit , 411–412T (1919).
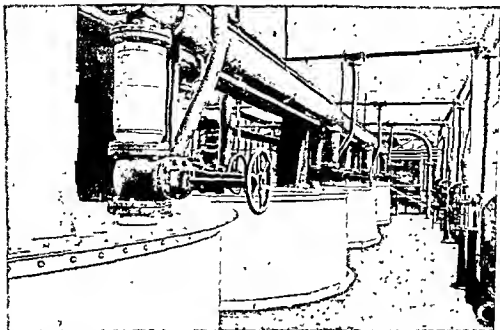
Fig 48—Mash tuns for mixing degermed corn meal and water. *(Courtesy of G. O. Lines, Commercial Solvents Corp)*
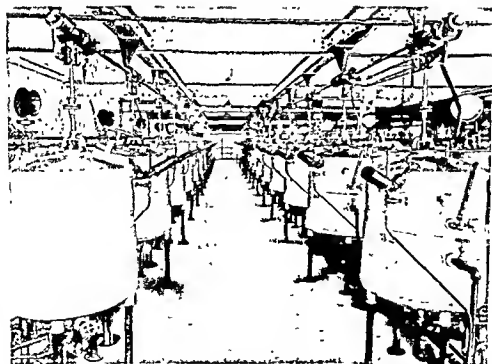
Fig 49—Propagation of bacteria. Eighty-gallon culture tanks *(Courtesy of O. O. Lines, Commercial Solvents Corp)*

It is essential to sterilize all the fermenters, pipes, pipe connection and equipment with which the mash or butanol organisms may come into contact, for contamination in this fermentation is usually a very serious matter that may involve considerable losses.

Culture
of Clostridium
acetobutylicum

Low-grade corn ──→ Degerming process

Spores on soil

Screened

Feed

Shocked ←── Reground ←── Roller mill

Magnetic separator

Oil

Coarse ←── Separator ── Husk ──→ Dried ──→ Feed

Test tube culture
in glass

Fines

Starch

500-cc culture
in glass ←── Special mash
tuns

Scale hoppers

6000-cc culture
in glass

Mash tuns

50-gallion culture
(mash made in autoclave)

Cookers (sterilizers)

1000-gallon culture
in iron

Hot water

Coolers

Cooling water

Hydrogen

Fermenters (50 000 gal.) ──→ Gas

Carbon dioxide

Reservoir (2.5 per cent solvents)

Continuous beer stills

Crude
Spirit (50 per cent solvents)

Waste water

Screens ──→ Solids ──→ Feed

Water to waste

Fractionating
columns

Mixed fractions

Acetone
(30 parts)

Ethyl
alcohol
(10 parts)

n-Butanol
(60 parts)

Fig. 50 — Flow sheet for butanol production. [Courtesy of D. H. Killeffer, Ind. Eng. Chem., 19: 46 (1927).]

Details of the Process. *Microorganisms.*—*Clostridium acetobutylicum* McCoy, Fred, Peterson, and Hastings; *Cl. butyricum* Prazmowski;[1] and

[1] "Bergey's Manual of Determinative Bacteriology," 6th ed., The Williams & Wilkins Company, Baltimore, 1948.

other bacteria may be used for the production of neutral solvents by fermentation.

There is, of course, a fairly large number of organisms, some non-sporeforming, which will produce butanol, but these bacteria would be unsatisfactory for commercial practice for various reasons Some are pathogenic, some will produce only small quantities or traces of solvents, some are slow fermenters, some will not attack starch, some are aerobic, and some lack vigorous cultural characteristics

Berthelot and Ossart stated that of the many bacteria isolated by them, aerobic and facultative, which produced acetone, only a few produced it in a quantity that was at all comparable with that of the anaerobic organisms used in industry

A cultural study of the acetone-butyl alcohol organism embodying such factors as reaction of the medium, temperature, reduction of dyes, and fermentability of carbohydrates was carried out by McCoy, Fred, Peterson, and Hastings.[2] They suggested *Cl. acetobutylicum* as a suitable name for the acetone-butanol organism of Weizmann.

Cl. acetobutylicum has now been adopted as the name of the species of *Clostridium* that produces relatively large quantities of acetone and butanol from starchy materials. Hence the Weizmann bacillus would properly be designated as *Cl acetobutylicum* McCoy, Fred, Peterson, and Hastings [3]

McCoy[4] and her associates have classified the motile and spore-forming butyric anaerobes of fermentation as a nonpathogenic subgroup of the genus *Clostridium*, which yields larger amounts of butyric acid or neutral products than the pathogenic clostridia, gives the granulose reaction and is catalase negative. The butyric anaerobes were divided into two general groups:

Group 1.   Acid end products, chiefly butyric and acetic acids—the true butyric anaerobes.

Subtype *A*.   *Cl pasteurianum* type—nonstarch-fermenting bacteria.

Subtype *B*.   *B saccharobutyricus* type—starch-fermenting clostridia and occasionally plectridia

Subtype *C*   Starch-fermenting plectridia

[1] BERTHELOT, A , et E. OSSART, *Compt rend*, **173**: 792 (1921)

[2] McCoy, E., E B. FRED, W H PETERSON, and E G HASTINGS, *Jour Infectious Diseases*, **39**: 457 (1926).

[3] "Bergey's Manual of Determinative Bacteriology," 6th ed., The Williams & Wilkins Company, Baltimore, 1948.

[4] McCoy, FRED, PETERSON, and HASTINGS, *op cit*, **46**: 118 (1930).

Group 2. Butyric and acetic acids as intermediate products, followed by neutral products (alcohols, or alcohol plus acetone)—the butanol organisms of industry.

Culture Methods.—The continued transfer of a seed culture in the usual media results in the culture becoming sluggish, with a corresponding decreased yield of solvents. This observation has been made independently by several different workers.

It has been shown that the most prodigious producers of solvents are those cultures whose spores are in general the most heat resistant

"Heat shocking" is a method wherein the vegetative cells and the weaker spores of a culture are destroyed. By subjecting a culture of the organism, in which the development of spores has been favored, to a temperature of 100°C. for 1 to 2 min., heat shocking is effected

It must be borne in mind that the severity of the treatment bears a direct relation to the temperature employed, the size and nature of the tube containing the seed culture, and the characteristics of the medium If one is to use thin-walled wafer tubing and a very small amount of the culture, it is obvious that neither the time of shocking nor the temperature should be excessive, since even the resistant spores may be destroyed

Weyer and Rettger "pasteurized" their butanol cultures in capillary tubes, which were 5 cm. in length. The tubes were filled with saline suspensions of the spores and plunged into water at 100°C. An exposure of 45 sec. to this temperature was the limit of the tolerance of the spores The capillary tubes were cooled quickly after the heat treatment.

Alternate heat shocking, or pasteurizing, and successive subculturing are commonly used to activate a culture. The medium containing the seed culture is allowed to stand at room temperature for a few days to encourage sporulation. New medium is inoculated from the spore-containing culture and heat shocked. The surviving pores are permitted to germinate under favorable conditions and subcultures are made successively at daily intervals for 4 to 7 days. At the end of this time the culture is again permitted to stand at room temperature to encourage spore formation. The cycle is then repeated—heat treatment, repeated subculturing, rest for sporulation, heat shocking, and so on.

Weizmann advocated heat treating a culture 100 to 150 times in order to improve its fermenting ability.

Underkofler and his coworkers[1] have shown that it is impossible to obtain good sporulation from sugar media when using ordinary culture tubes in the usual manner. They have demonstrated that sporulation could be successfully induced, however, provided the culture-containing

[1] UNDERKOFLER, L. A., L. M. CHRISTENSEN, and E I. FULMER, *Ind. Eng Chem*, 28 : 350 (1936).

medium was poured aseptically into a sterile flask of such size that the medium was exposed in a thin layer to air, or was dried on sterile soil.

Spores of *B. granulobacter pectinovorum*, grown in maize mash, retained their ability to produce acetone after storage for at least seven years in sealed glass tubes, according to Fowler and Subramanyan.[1] The spores were subcultured successfully in jawari mash (*Andropogon sorghum*).

In a study of six different strains of *Cl. acetobutylicum* (Weizmann), Weyer and Rettger (1927) brought out several facts of major importance. Storage of spores for a period of over 6 months apparently decreased their power of producing solvents. It was possible, however, to rejuvenate the cultures by alternate pasteurization and subculturing, for pasteurization destroyed the vegetative forms and the weaker spores. The most vigorous strains for fermentation appeared to be the most active sporeformers.

Cl. acetobutylicum (Weizmann) was rather susceptible to various antiseptic and germicidal substances. Spores withstood successfully the effects of a 2 5 per cent butyl resorcinol solution for 24 hr., but spores of *B. mesentericus* were destroyed or inactivated. The apparently selective action of the butyl resorcinol was believed to be due to the fact that the Weizmann organism has a certain tolerance for the butyl radicle.

Raw Materials—A rather wide variety of raw materials may be used to supply the carbohydrates and nutrient substances required to ensure a satisfactory fermentation. Starches rendered soluble by preliminary treatment, hydrolyzed starches, disaccharides, hexoses, pentoses, molasses, and sugar sirups have been used under different conditions. Corn is, of course, a readily available and easily fermented source of raw material in this country and elsewhere. Rice, jawari, bajra, and tapioca starches, peanut and oat hulls; corncobs; horse chestnuts; arabinose; and xylose have been fermented by butanol organisms with varying degree of success. Underkofler and others[2] have shown experimentally that as much as 80 per cent of corn meal may be replaced by sucrose or glucose without sacrificing high yields. Xylose may replace corn meal to the extent of 40 per cent. Thus it is possible to ferment along with corn meal materials which, by themselves, would produce small yields or be fermented with some difficulty. This is a sound practice from the point of view of conservation and economies.

Sjolander and his coworkers[3] have shown that butanol, acetone, and

[1] FOWLER, G J , V SUBRAMANYAN, *Jour Indian Inst Sci*, 8A 71 (1925)

[2] UNDERKOFLER, CHRISTENSEN, and FULMER, *loc cit*

[3] SJOLANDER, N O, A F LANGLYKKE, and W H PETERSON, Butyl Alcohol Fermentations of Wood Sugar, *Ind Eng Chem*, 30: 1251 (1938)

ethanol may be successfully produced from wood sugars by *Cl. felsineum* and *Cl. butylicum.*

Nitrogen Requirements.—According to Wilson and his associates,[1] *Cl acetobutylicum* was able to utilize protein, peptone, or aminoids (completely degraded proteins) as sources of nitrogen.

There were only slight changes in the ratio of solvents resultant from the use of nitrogen in the different forms. When peptone was the source
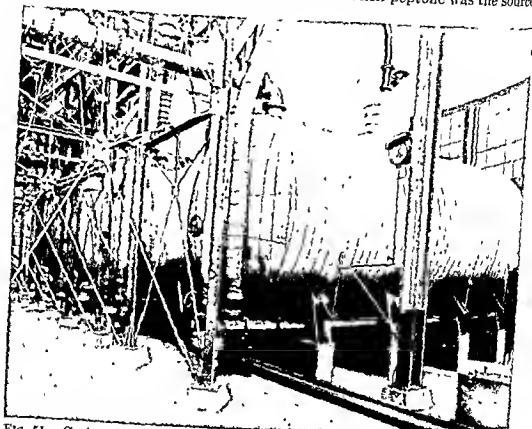
Fig. 51.—Cookers for sterilizing the corn meal and water. (Courtesy of G. O. Lines, Commercial Solvents Corp.)

of nitrogen, more acetone was produced with less ethanol; when beef aminoids were used, there was a small increase in the quantity of butanol. Large amounts of ammonium salts of the mineral acids prevented fermentation or decreased yields on account of the acids liberated upon hydrolysis. Mashes containing a deficiency of nitrogen were decidedly abnormal from the point of view of yield of solvents.

Ammonium salts or single amino acids do not serve as satisfactory sources of nitrogen in semisynthetic media.

Some Substances Essential for Normal Yields of Solvents.—Speakman, working with *B. granulobacter pectinovorum,* found that no growth

[1] Wilson, P. W., W. H. Peterson, and E. B. Fred, *Jour. Bact*, 19: 231 (1930).

resulted when the sole source of nitrogen in a mash was ammonium salts or a single amino acid   Weyer and Rettger[1] substantiated Speakman's conclusions and stated that a complex nitrogen supply, which may be furnished by proteins and commercial peptones, is necessary for growth of *Cl. acetobutylicum* and subsequent fermentation.

Working with *Cl acetobutylicum* (Weizmann), Weinstein and Rettger[2] found that a prolamine-containing substance was necessary for the production of normal amounts of acetone and butanol by fermentation. Hydrolyzed cottonseed hulls, peanut hulls, corncobs, and the like yielded normal amounts of acetone but no butanol. The addition of a prolamine-containing substance, yellow corn, stimulated the production of normal amounts of both solvents   The yields of solvents were higher than those obtained from corn alone or from the hydrolyzed product alone. It was ascertained that prolamine did not act as a catalyst. An alcohol-soluble protein was found to be necessary for the production of appreciable amounts of butanol from Robinson's medium

On the other hand, Weizmann and Rosenfeld (1937) state that complex proteins, such as peptone or prolamines, are not necessary for a normal butanol-acetone fermentation. They have shown that asparagine in the presence of an activator will produce a normal fermentation in a semisynthetic medium   The activator is a compound of low molecular weight of unknown composition   It is possibly not a single substance. According to Weizmann, the activator is probably of the nature of a coenzyme, which may play an essential part as a hydrogen carrier as well as favor growth of bacteria. Insufficient anaerobiosis, absence of the activator, or both, led to the production of acids, principally butyric acid. In the absence of asparagine and the activator, the fermentation may not proceed at all, or very slowly   The activator is found in seeds, green plants, and in yeast. It is thermostable. (For a further discussion of this subject, the reader is referred to the publication by Weizmann and his associate)

Brown and his associates[3] obtained normal yields of neutral solvents by culturing butanol-acetone organisms in a medium that contained glucose, hydrolyzed casein, tryptophane, ammonium sulphate, mineral salts, and an "acidic ether-soluble extract" obtained from Difco yeast extract

McDaniel, Woolley, and Peterson[4] have partially purified an accessory

[1] Weyer, E R, and L F. Rettger, *Jour Bact*, **14:** 399 (1927).

[2] Weinstein, L, and L. F. Rettger, *Jour Bact*, **23:** 74 (1932).

[3] Brown, R W., H G Wood, and C H Werkman, *Jour Bact*, **35:** 206 (1938)

[4] McDaniel, L. E, D W. Woolley, and W. H. Peterson, *Jour Bact*, **37:** 259 (1939)

substance, which together with glucose, asparagine, and inorganic salts permitted growth of *Cl. acetobutylicum* and of *Cl. butylicum* in a medium. The stimulating substance could not be replaced by beta-alanine, indole acetic acid, inositol, nicotinic acid amide, pantothenic acid, pimelic acid, riboflavine, sporogenes growth factor, vitamin B_1, vitamin B_6, or by a "mixture of all of the naturally occurring amino acids" (hydroxy-glutamic acid excepted). It was stable to bromination, to steaming in normal alkali, and to autoclaving.[1]

Biotin and a factor found in yeast extract are necessary for *Cl acetobutylicum* in the acetone-butanol fermentation, according to Oxford, Lampen, and Peterson (1940).

Substances Stimulating the Production of Butanol by Certain Butyric Acid Bacteria.—In 1934, Tatum, Peterson, and Fred[2] reported the presence of an unknown substance in potatoes, yams, cabbage, lettuce, alfalfa, navy and soybeans, malt sprouts, and wheat middlings that caused an increased fermentation of starch and a marked increase in the yield of butanol through the action of certain butyric acid bacteria. No changes were effected in the quantities of ethyl alcohol and acetone produced. Barley, corn, oats, and rice were poor sources of the stimulating substance and sometimes were found to contain none of it.

Later, Tatum and his associates[3] identified *l*-asparagine ($H_2NOC\cdot CH_2\cdot CH(NH_2)\cdot COOH$) as the substance producing the stimulatory action. Further research disclosed that *l*-aspartic ($HOOC\cdot CH_2\cdot CH(NH_2)\cdot COOH$) and *d*-glutamic ($HOOC\cdot CH_2\cdot CH_2\cdot CH(NH_2)\cdot COOH$) acids, which are dicarboxylic amino acids, produced effects equivalent to those of *l*-asparagine when these acids were supplemented with molar equivalents of ammonium sulphate. The optimum concentration of these stimulants was 60 mg. per 100 cc. of 4 per cent corn-meal mash, although lower concentrations produced stimulation.

Asparagine may be hydrolyzed by the enzyme asparaginase to yield aspartic acid and ammonia.

$$
\begin{array}{ccc}
\text{CO·NH}_2 & & \text{COOH} \\
| & & | \\
\text{CH}_2 & \xrightarrow[+\text{H}_2\text{O}]{\text{Asparaginase}} & \text{CH}_2 \\
| & & | \qquad +\text{NH}_3 \\
\text{H}_2\text{N CH} & & \text{H}_2\text{N CH} \\
| & & | \\
\text{COOH} & & \text{COOH} \\
\text{Asparagine} & & \text{Aspartic acid}
\end{array}
$$
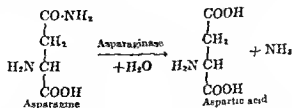
Asparaginase is secreted by some yeasts and bacteria and has an optimum pH of 8.

[1] *Ibid.*
[2] Tatum, E. L., W. H. Peterson, and E. B. Fred, *Jour. Bact.*, 27 : 207 (1934).
[3] *Ibid*, 29 : 563 (1935).

Ammonium malate ($H_4NOOC\cdot CH_2\cdot CHOH\cdot COONH_4$) and ammonium succinate ($H_4NOOC\cdot CH_2\cdot CH_2\cdot COONH_4$) were likewise found to stimulate the fermentation of starch by certain, but not all, butyric acid bacteria and to cause increases in the yields of butanol, but these substances were less effective than *l*-asparagine.

Optimum Conditions for Fermentation. *Temperature.*—The most favorable temperature range is 37 to 42°C Since the neutral solvents are volatile at these temperatures, especially acetone, it is necessary to take precautions to avoid losses during the fermentation process.

Oxygen Relationship.—Since the organisms best suited to the production of acetone and butanol are anaerobic in nature, the highest yields will be obtained when anaerobiosis is maintained.

pH.—Growth may be obtained in corn mashes between a pH of 4 7 and 8 by the butanol organisms, but there is a low production of solvents at both of the extremes. The pH range of 5 to 7 is in most cases satisfactory

The assertion that a given concentration of hydrogen ions is completely inhibitory at all times or is capable of producing any given degree of inhibition, is not justified, according to Wynne,[1] who carried out experiments upon the inhibition of the acetone-butanol fermentation by acids The inhibition of fermentation was in the following order: nonylic $\geq$ caprylic > heptylic > formic > isocaproic = caproic > valeric = isovaleric > isobutyric = butyric $\geq$ propionic = acetic This order is similar to that of the speed of penetration of the acids into the living cells The pH necessary for pyruvic acid to inhibit is 3 2 which is lower than for most acids.

Effect of Calcium Carbonate.—In general, the addition of calcium carbonate to the acetone-butanol fermentation medium causes a decrease in the yields of acetone and butanol in proportion to the amount of calcium carbonate added The volatile acid content, butyric and acetic acids, principally, is increased, while alcohol formation is suppressed

Concentration of Raw Material.—The exact concentration of the carbohydrate-containing substance to be used in a given fermentation must be determined by experimentation, unless facts concerning the fermentation organism are already known Mashes containing 3 to 10 per cent concentrations of corn meal are readily fermented with high yields.

Production from Other Substrates.—Corn and molasses have been the principal raw materials used for the production of butanol and acetone by fermentation in this country because of their availability, ease of handling, and relatively low price However, there are other carbohydrate sources that offer potentialities Some of these will be mentioned briefly

[1] WYNNE, A M , *Jour Bact*, **22**: 209 (1931)
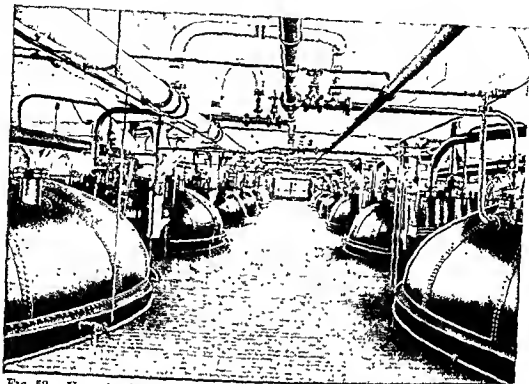
Fig. 52.—Upper level of 50,000-gal. fermentation tanks.   (Courtesy of G. G. Lines, Commercial Solvents Corp.)

Fig. 53.—Lower level of 50,000-gal. fermentation tanks.   (Courtesy of G. O. Lines, Commercial Solvents Corp.)

Production from Waste Sulphite Liquor.—The production of acetone and butanol from waste sulphite liquors has been studied by Wiley and associates.[1] They found that good yields of the solvents were obtained when the sulphite liquor was prepared by precipitating the sulphur dioxide with calcium hydroxide at a pH of 10 as calcium sulphite; by precipitating the lignin by adjusting the pH to 11.5 with additional calcium hydroxide; by precipitating the excess calcium with 1 per cent by weight of sodium sulphate before neutralizing; and by neutralizing the liquor to a pH of 5.8 with sulphuric acid. The precipitates formed during the various treatments were removed. The following nutrients were found to be satisfactory: 0.05 per cent diammonium hydrogen phosphate $(NH_4)_2HPO_4$, 0.10 per cent molasses, and 0.10 per cent calcium carbonate. *Clostridium butylicum* (Fitz strain) was found to be the most suitable organism. From 70 to 80 per cent of the total reducing substances found in the sulphite liquor were fermentable, and from 25 to 30 per cent of the sugars were fermented to neutral solvents in the following proportions: 75 parts of butanol, 20 parts of acetone, and 5 parts of ethanol.

Production from Wood Sugar.—Leonard, Peterson, and Ritter[2] found that 24 to 38 per cent of the wood sugars fermented were converted to neutral solvents by *Cl. butylicum* (No. 39 of the University of Wisconsin collection). The hydrolyzates were distilled to remove the furfural and neutralized to a pH of 6.5 with lime. It was found that sugar solutions produced by very mild or very vigorous conditions of hydrolysis were not easily fermented. Sugar concentrations up to 3 per cent were completely utilized.

Production from Xylose Saccharification Liquors from Corncobs.—Butyl alcohol may be produced in satisfactory yields from the xylose saccharification liquors obtained from corncobs in the first stage of the two-stage process described by Dunning and Lathrop (1945), after treatment with powdered iron or activated carbon, according to a report made by Langlykke, Van Lanen, and Fraser (1948).

The following nutrients were used in the media. 0.2 per cent corn steep liquor (dry basis), 0.5 per cent calcium carbonate, 0.3 per cent ammonium sulphate, and 0.06 per cent dibasic ammonium phosphate. The sugar source was usually diluted to produce a final concentration of 5 to 6 per cent of total sugar. The media were adjusted to pH 6.5 prior to sterilization. Incubation was at 37°C.

[1] WILEY, A. J., M. J. JOHNSON, E. McCOY, and W. H. PETERSON, *Ind. Eng. Chem.*, 25: 606 (1941).

[2] LEONARD, R. H., W. H. PETERSON, and G. J. RITTER, *Ind. Eng. Chem.*, 39: 1413 (1947).

Satisfactory fermentation of saccharification liquors were obtained when they were treated by the addition of 0.5 per cent of reduced iron or iron powder. The use of iron filings ground to pass a 325-mesh screen was suitable as a substitute for reduced iron. The iron removed copper from the hydrolyzates and also lowered their oxidation-reduction potentials. Copper in concentrations greater than 15 p.p.m. was found to be inhibitory to the butanol organism [culture A-14 (NRRL B-594) of McCoy's collection at the University of Wisconsin] under the conditions of fermentation.

The treatment of the hydrolyzate with 15 per cent or more activated carbon (on the basis of the sugar) was also a satisfactory means for producing good yields.

Yields of 30.6 per cent of solvent were obtained on the average, of which 61.7 per cent was butanol, 31.8 per cent was acetone, and 6.5 per cent was ethanol.

*Production from Jerusalem Artichokes—*The production of butanol and acetone from Jerusalem artichokes has been studied by Wendland, Fulmer, and Underkofler.[1] The ground chips were diluted with water to yield a reducing sugar content of 5 per cent. The suspension was mildly hydrolyzed, for 1 hr. at 80°C. after adjustment of the pH to 1.75 with hydrochloric acid or to 1.50 with sulphuric acid. The hydrolyzate was neutralized to a pH of 5.5 to 6.0 with sodium hydroxide. It was found necessary to add corn meal, soybean meal, or other nutrients to the hydrolyzate in order to obtain maximum yields with *Cl acetobutylicum.*

*Production from Cassava.—*Cassava only is not a good substrate for acetone-butanol production, according to Banzon.[2] However, good yields could be obtained from a mixture of 20 per cent or more of corn and 80 per cent or less of cassava. Likewise, yields of solvents comparable to those obtained from corn were secured when about 5 per cent of shrimp powder, corn gluten meal, and soybean flour were used as constituents of the mash.

*Yield of End Products.—*In the normal butanol-acetone fermentation, neutral solvents are formed from glucose in the ratio of 6 parts of *n*-butanol, 3 parts of acetone, and 1 part of ethyl alcohol. Sucrose, levulose, and xylose yield normal ratios of butanol, acetone, and ethanol, but arabinose, a 5-carbon sugar, yields the solvents in a ratio of approximately 5:4:1 instead of the normal 6.3:1 ratio. These facts are illustrated in the following table.

[1] Wendland, R. T., E. I. Fulmer, and L. A. Underkofler, *Ind. Eng. Chem.*, **33:** 1078 (1941).

[2] Banzon, J. R., *Iowa State Coll. Jour. Sci.*, **16:** 15 (1941).

TABLE 71—AVERAGE SOLVENT RATIO FOR THE ACETONE-BUTANOL FERMENTATION OF SUGARS[1]

Investigators	Sugar	No of fermentations	Average solvent ratio		
			Butanol	Acetone	Ethanol
Peterson, Fred, and Schmidt[2]	Glucose	2	59	31	10
	Xylose	3	61	29	10
	Arabinose	2	47	43	10
Johnson, Peterson, and Fred	Glucose	18	60	28	12
	Arabinose	4	48	39	13

[1] UNDERKOFLER, L A , and J E HUNTER, JR, *Ind Eng Chem*, 30: 480 (1938).
[2] Ratios calculated from the data, assuming 10 per cent ethanol.

FIG 54 —Distillation equipment   *(Courtesy of G O Lanes, Commercial Solvents Corp)*

From 3 lb of starch, 1 lb of mixed solvents may be obtained when *Cl acetobutylicum* is used as the fermentation organism

At the Royal Naval Cordite Factory at Holton Heath, England, 163 lb of n-butanol, 70 lb of acetone, **107 lb** of carbon dioxide, 11 lb of

hydrogen, and 12 lb. of residual acid were obtained from 1,000 lb. of maize, which contained 650 lb. of starch.[1]

The gases produced during fermentation weigh over 1.5 times as much as the neutral solvents formed. Were these gases allowed to go to waste, as they were at one time, the losses would be great.

Acetylmethylcarbinol.—In 1927, Wilson and his associates[2] showed that acetylmethylcarbinol was produced in the acetone-butanol fermentation by *C. acetobutylicum* (Weizmann's bacillus) as a regular end product. Acetylmethylcarbinol was formed concurrently with acetic and butyric acids. Pyruvic acid when added to a fermenting mash was fermented to acetic acid, acetone, and acetylmethylcarbinol chiefly (Peterson and Johnson, 1933). The addition of phosphates to a mash increased the production of acetylmethylcarbinol, while added proteins decreased the yield. Ordinarily 300 to 400 mg. per liter of acetylmethylcarbinol are produced in the butanol fermentation.

Yellow Oil.—In the butanol fermentation, so-called "yellow oil" makes up 0.5 to 1 per cent of the total yield of solvents. Marvel and Broderick[3] showed that the high boiling yellow oil was a complex mixture of *n*-butyl alcohol, active amyl alcohol, isoamyl alcohol, *n*-hexyl alcohol, and the *n*-butyric, caprylic, and capric esters of these alcohols.

Uses for the Products of Fermentation.—Butanol has its most important use in the manufacture of lacquers, which are utilized on automobiles, airplanes, furniture, toys, and many other articles. Large quantities of butanol and its derivatives are used in other industrial processes. Acetone is used in the manufacture of artificial silk and leather, photographic film, airplane dopes, cements, and other products. The accompanying table shows some detailed uses for the chemical products manufactured from corn.

The weight of gases evolved during fermentation is over $1\frac{1}{2}$ times greater than the weight of solvents. Hence, in order to prevent enormous wastes, uses for the hydrogen and carbon dioxide gases have been developed. Methanol (CH_3OH) is synthesized from hydrogen and carbon dioxide gases. These gases are passed through a solvent recovery plant to remove and recover any solvents that may have been carried over with the gases during the fermentation. Part of the carbon dioxide is then removed by "scrubbing" the gases with water under pressure. The purified gases are then forced through a porous, catalytic mixture at a high temperature and at a pressure of about 4,500 lb. to produce

[1] REILLY, HICKINBOTTOM, HENLEY, and THAYSEN, *loc. cit.*
[2] WILSON, P. W., W. H. PETERSON, and E. B. FRED, *Jour. Biol. Chem.*, **74:** 495 (1927).
[3] MARVEL, C. S., and A. E. BRODERICK, *Jour. Am. Chem. Soc.*, **47:** 3045 (1925).

TABLE 72.—CHEMICALS FROM CORN AND THEIR USES[1]

ACETONE	CORN OIL	BUTANOL	ETHYL ALCOHOL	METHANOL
	CORN GERM MEAL		CARBON DIOXIDE	

ACETYLENE SOLVENT	PHOTOGRAPHIC FILMS	LACQUERS	PHOTOGRAPHIC FILMS	METAL CLEANERS	FORMALDEHYDE	METHYL SALICYLATE
CELLULOSE ACETATE SILK	PYROXYLIN PLASTICS	SPIRIT VARNISHES	ARTIFICIAL AND PATENT LEATHER	ALCOHOL DENATURANT	SYNTHETIC RESINS	PAINT & VARNISH REMOVER
LINIMENTS	CELLULOSE ACETATE	DYE SOLVENT	DRUG EXTRACTION	DEHYDRATER	EMBALMING FLUID	DYE SOLVENT
AIRPLANE DOPES	NITROCELLULOSE CEMENTS	PENETRATING OILS	SOLDERING FLUX	DE-FROTHER	ANTIFREEZE	ALCOHOL DENATURANT
PAINT & VARNISH REMOVER	DEWAXING OILS	RUST REMOVERS	SYNTHETIC RESINS	DRY CLEANING	SPIRIT VARNISHES	DEWAXING GUMS
ALCOHOL DENATURANT	ARTIFICIAL LEATHER	BUTYL PROPIONATE	BUTYL XANTHATE	BUTYL CELLOSOLVE	ESSENTIAL OIL SOLVENT	DRY CLEANING
SYNTHETIC RESINS	SMOKELESS POWDER	BUTYL ALDEHYDE	BUTYL ACETATE	DIBUTYL PHTHALATE	LITHOGRAPHIC WORK	PYROXYLIN PLASTICS
EXTRACTION	DRUGS	RUBBER ACCELERATOR	LACQUERS	PLASTICIZER	METHYL ANTHRANILATE	METHYL CHLORIDE
CHLOROFORM	IODOFORM	BUTYL CHLORIDE	BUTYL STEARATE	BUTYL LACTATE	DIMETHYL ANILINE	DIMETHYL AMINE
DIACETONE ALCOHOL		ALCOHOL DENATURANT	WATERPROOFING COMPOUNDS	BRUSHING LACQUERS	MONOMETHYL AMINE	
ANTIFREEZE	COMPRESSION FLUIDS	DIBUTYL TARTRATE	BUTYL ACETI RICINOLEATE		DEHAIRING HIDES	DYE INTERMEDIATE
		CELLULOSE ACETATE LACQUERS	EMULSIFICATION AGENT			

synthetic methanol. Ammonia may be synthesized from purified hydrogen and nitrogen gases, using a catalyst, while carbon dioxide may be used in dry-ice manufacture.

Bacterial Contaminants.—The most serious contaminants in the acetone-butanol fermentation are the lactic acid organisms. Lactic acid bacteria grow readily at the temperature of the butanol fermentation and under anaerobic conditions. They utilize the substrate and at the same time produce a pH unfavorable for butanol production. The high-acid-forming bacteria,[1] such as *Lactobacillus leichmannii*, are most injurious. Organisms such as *L. mannitopoeum* are likewise very undesirable contaminants *B. volutans* n. sp. Fleming, Thaysen,[2] a most serious contaminant, is a nonsporeforming organism that produces large amounts of lactic acid, traces of butyric and acetic acids, but no gas or alcohols It is a Gram-positive organism, possessing volutin granules that may be stained by methylene blue to a deep purple. This organism may be destroyed by heating it for 5 min. at 65°C. *Streptococcus lactis* also has been known to cause infections, but it does not usually produce serious trouble Fermentations continue to completion unless the organisms are present in large numbers in the mash before the butanol organisms have had opportunity to develop.

The presence of *B. globigii* in a butanol fermentation is a type of association that produces no apparent ill effect on the yield by *B. granulobacter*.[3]

Sporeformers of the *B. mesentericus* group may produce a red pigment in corn mashes[3] but otherwise produce no apparent harm.

Methods of Detecting Contamination—Various methods have been used to detect contamination in the fermenting mash.[4] Observation of variations in gas evolution or in the titrable-acidity curve, and the use of the microscope are the most common methods.

Perhaps the most sensitive indicator of contamination is gas evolution One accustomed to observing normal fermentations can readily ascertain irregularities in the evolution of gas. A marked reduction in the volume of gas evolved at a time when the rate of evolution should be increasing may be taken as an indication of contamination. Gas evolution may even cease.

By plotting the curve for titrable acidity, one has available accurate information concerning the progress of the fermentation. Any increase in acidity to abnormal amounts can be readily ascertained by an examina-

[1] Fred, E B., W. H. Peterson, and M. Mulvania, *Jour Bact*, **11**: 323 (1936).

[2] Thaysen, A C., *Jour Inst Brewing*, **27**: 529 (1921)

[3] Fred, E B., W H Peterson, and W. R. Carroll, *Jour Bact*, **10**: 97 (1925)

[4] Speakman, H B., and J F Phillips, *Jour. Bact.*, **9**: 183 (1924).

tion of the acid curve. After rising to a peak in 13 to 17 hr., the normal curve for titrable acidity drops at about the same rate to a new low point In case the mash is contaminated by lactic acid organisms, the curve for titrable acidity continues to rise after the normal maximum instead of returning to the new low point. In some industrial plants a practice was made to inoculate large mashes from starters only after the curve for titrable acidity had commenced to fall (The reader should realize
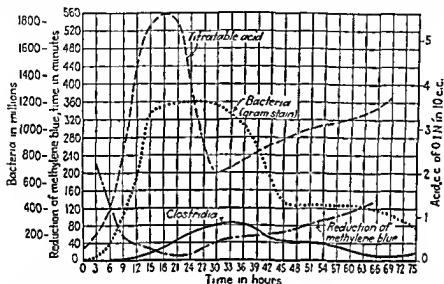
Fig 55—Number of bacteria, time of reduction of methylene blue, and acid production at different stages during fermentation. [*Courtesy of W H Peterson and E B, Fred, Ind Eng Chem,* 24· 237 (1932)]

that variations in the nature of the curve for titrable acidity sometimes occur even though there is an absence of infection)

The microscope has served as a useful tool in detecting contamination, yet it may fail to show contamination when either variation in gas evolution or titrable acidity may strongly indicate the presence of infective organisms. *The microscope is best used in conjunction with one of the foregoing methods rather than alone.*

Marked changes in the curve for pH would indicate unusual conditions in the fermenting mash. Changes in pH do not, however, furnish either as sensitive or as accurate signs of an abnormal fermentation as do abnormal variations in gas evolutions and in titrable-acidity curves.

Biochemistry of the Fermentation.—Speakman[1] divides the butanol fermentation into three phases, the length of each phase being governed by the curve for titrable acidity During the first phase, the titrable acidity increases rapidly to a maximum, usually in 13 to 17 hr. The

[1] SPEAKMAN H B, *Jour Biol Chem,* **41**: 319 (1920)

butanol organisms reproduce very rapidly during this period, for it is their enzymic activity that determines the progress of the fermentation Acetic and butyric acids are produced in varying quantities. Both hydrogen and carbon dioxide gases are produced in large amounts, the curve for total gas evolution following the titrable-acidity curve in general proportions but with a time lag. There is a drop in pH, which then tends to remain at a fairly constant level throughout[1] the rest of the fermentation, owing to the presence of buffers produced through the hydrolysis of the corn or other proteins.

The quantity of titrable acidity drops sharply during the second phase of fermentation to a value that is equal to approximately 50 per cent of the maximum. Coincidental with the drop in the quantity of titrable acidity, there is a rapid conversion of the acids to their corresponding solvents. Butyric acid is reduced to butanol, acetic acid is changed to acetone. The ratio of acetic to butyric acid varies during this period. Butyric acid disappears from the fermentation mash more rapidly than acetic acid. The rate of gas evolution increases quickly to a maximum as the titrable acidity drops from the peak. There is then a gradual diminution in the rate of gas evolution until the end of the fermentation.

The titrable acidity slowly increases in quantity during the third phase of the fermentation. There is a drop in the rate of solvent production until the fermentation ceases. The relative proportions of acetic and butyric acids continue to vary until at the end of the fermentation there is a greater amount of acetic acid.

Peterson and Fred[1] have carried out extensive research on the bacteriology and biochemistry of the acetone-butanol fermentation by Cl acetobutylicum. Figures 55 to 60 are reproduced (through their courtesy) from their excellent paper. The data from which the curves were constructed were determined by periodic analyses of the fermenting corn mashes, which at the start were of 6 per cent concentration. Results shown in Figs. 55 to 58 were obtained in the first experiment of Peterson and Fred, those of Fig. 59 in the second experiment, and those of Fig. 60 in the third experiment. A study of these figures will yield much valuable information.

Only small amounts of peptides or amino acids are formed from corn mashes during the first 12 hr. of the fermentation and not much during the first 24 hr., according to Peterson and his associates.[2] The amino acid and peptide content of the mash increases rapidly during the next 24 hr., however.

    [1] PETERSON, W. H., and E. B. FRED, Ind. Eng. Chem., 24: 237 (1932).
    [2] PETERSON, W. H., E. B. FRED, and B. P. DOMOGALLA, Jour. Am. Chem. Soc., 46: 2086 (1924).
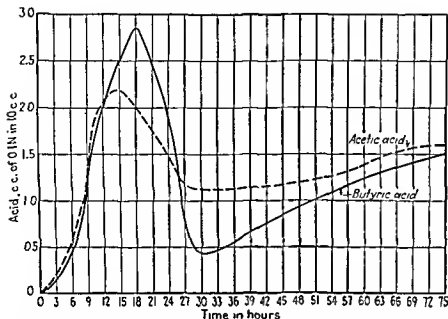
Fig. 56—Nature of volatile acids formed by *Cl. acetobutylicum.* [*Courtesy of W H Peterson and E B. Fred, Ind Eng Chem , 24·237 (1932).*]
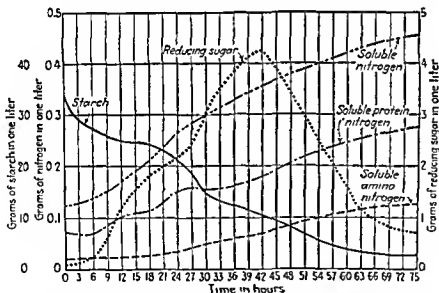
Fig 57—Changes in carbohydrates and forms of nitrogen caused by *Cl acetobutylicum.* [*Courtesy of W H Peterson and E B. Fred, Ind Eng Chem , 24 237 (1932)]*

Fulton, Peterson, and Fred[1] state that from 15 to 60 per cent of the total nitrogen of native proteins is rendered soluble during the fermenta-

[1] FULTON, H L , W H PETERSON, and E B FRED, *Centr Bakt Parasitenk ,* Abt II 27·1 (1926)
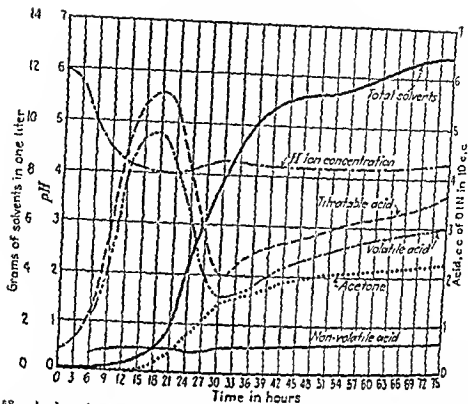
Fig. 58 —Acid production and solvent formation [Courtesy of W. H. Peterson and E. B. Fred, Ind. Eng. Chem., 24. 237 (1932)]
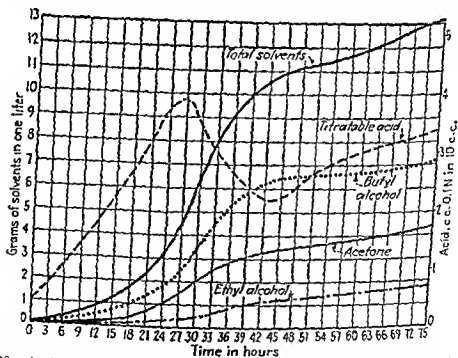
Fig. 59 —Acid production and solvent formation. [Courtesy of W. H. Peterson and E. B. Fred, Ind. Eng. Chem., 24: 237 (1932)]
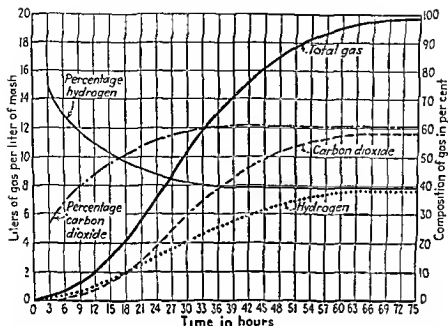
FIG 60—Production of gas in butyl alcohol fermentation [*Courtesy of W. H. Peterson and E. B. Fred, Ind. Eng. Chem., 24: 237 (1932).*]

tion. The soluble derivatives of protein (proteoses, peptones, peptides, and amino acids) and nonamino nitrogen constitute the soluble nitrogen. Peptide nitrogen is usually the largest single source of soluble nitrogen, constituting 9 to 28 per cent of the total. Amino nitrogen is generally present in quantities of 7 to 18 per cent, depending upon the type of protein being fermented, animal proteins yielding rather high quantities of amino nitrogen.

The products formed by the hydrolysis of proteins, a process that appears to progress well at a pH of about 5.5, regulate the pH of the medium through buffer action. Butyric and acetic acids are but slightly dissociated. Thus high titrable acidity produces very little change in the pH of the medium.

Factors that cause an increase in acetone frequently produce a decrease in the quantity of ethanol formed. Proteolysis, acid production, and the quantity of solvents produced are affected by the carbohydrate-protein ratio of the mash. Low carbohydrate-protein ratios favor increased yields of acetone and decreased yields of ethanol. Opposite effects result from high carbohydrate-protein ratios. In general, high yields of solvents may be obtained with carbohydrate-protein ratios of 5 to 10 (Fulton, Peterson, and Fred, 1926). High titrable acidity is generally associated with a high yield of acetone.

Peterson and Johnson[1] found that *Cl. acetobutylicum* fermented added acetoacetic acid most rapidly during the period when solvents were being formed at a maximum rate. As much as 3.2 g. of acetoacetic acid per liter has been fermented during 8 hr. in some fermentations, the acid being decarboxylated to acetone.

Formic acid added to a fermenting mash was destroyed by *Cl. acetobutylicum.*[2] This fact strengthens the hypothesis that formic acid is the precursor of hydrogen and some of the carbon dioxide gas.

For further discussion of the biochemistry of the butanol fermentation, the reader is referred to the section dealing with the mechanism of the fermentation.

Mechanism for the Formation of End Products.—Before attempting to formulate a scheme to show the origin of the products of a fermentation, it is necessary to assemble some of the known facts A careful study of the charts concerned with the biochemistry of the butanol fermentation will be most fruitful in this connection.

During the butanol-acetone fermentation, butyric acid disappears more rapidly than acetic acid, a fact in accord with the production of a greater proportion of butanol than acetone (Reilly, Hickinbottom, Henley, and Thaysen). The decrease in butyric acid is coincidental with the increase in butanol. Additions of butyric acid to a fermenting mash increase the yield of butanol (Speakman and others). Butyric acid is reduced to butanol by cell suspensions when in the presence of glucose but not by pyruvate. It is thus probable that glucose, or possibly triose-phosphate, is the reducing agent for butyric acid, according to Davies (1942). Butyric acid is nearly always found in fermentations when butanol occurs.

Acetic acid disappears from the fermentation medium more slowly than butyric acid, a fact agreeing with the formation of a smaller amount of acetone than butanol The addition of acetic acid to a fermenting mash increases the acetone content, but, according to Speakman, does not cause any change in the yield of butanol. Acetic acid, acetoacetic acid, and pyruvic acid increase the quantity of acetone produced when added to the fermentation (Johnson, Peterson, and Fred, 1933). Propionic acid is reduced to propyl alcohol.

Davies (1943) has purified and described the properties of the acetoacetic acid decarboxylase of *Cl. acetobutylicum.* The enzyme was specific for acetoacetic acid and had an optimum activity at pH 5.0.

Pyruvic acid is fermented to acetic acid, acetone, and acetylmethyl-

[1] Peterson, W H, and M J Johnson, *Jour. Bact*, 25. 69 (1933)
[2] Stiles, H. R, W. H Peterson, and E. B. Fred, *Jour Biol Chem*, 84: 437 (1929).

carbinol when added to the fermented mash, but methylglyoxal and aldol are toxic even in small amounts

Calcium carbonate in the fermentation mash results in a suppression of butanol and acetone production but causes an increase in the quantities of butyric and acetic acids.

A workable scheme must show the derivation of all the end products in relatively correct proportions. It must be in accord with established facts of fermentation chemistry. Study of the following schemes will show how closely these principles are approached.

Several schemes have been suggested to explain the origin of the end products of a normal butanol-acetone fermentation. Fitz suggested the formation of 4-carbon compounds from 2-carbon compounds by a process of condensation.

Speakman[1] was one of the first to suggest a detailed scheme for the mechanism of the butanol-acetone fermentation. According to him butyric and acetic acids were formed by cleavages and oxidations of the sugar molecule. These acids were then reduced in part to the corresponding solvents.

Neuberg and Arinstein[2] suggested that butyric acid and butanol were formed from glucose and glycerol through the intermediate stages of pyruvic acid and pyruvic aldol.

Pyruvic acid is produced by fermentation from glucose, glycerol, or lactic acid by various microorganisms. But Neuberg and Arinstein were unable to increase the yields of butyric acid or butanol through the addition of a pyruvate to a fermenting medium. They did, however, secure increases of acetic and formic acids in accordance with the following equation:

$$CH_3 CO COOH + H_2O \rightarrow CH_3 COOH + HCOOH$$
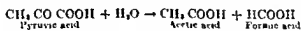
Neuberg and Arinstein therefore concluded that butyric acid and butanol did not arise as a result of the condensation of acetaldehyde and carbon dioxide. The addition of pyruvic aldol resulted in increased yields of butyric acid. On the basis of these facts, they formulated their schemes.

Various objections have been raised in connection with the schemes suggested by Neuberg and Arinstein. The organism that they used, *B butylicus*, under optimum conditions produced very little butanol The fact that added pyruvic aldol was fermented to butyric acid does

[1] SPEAKMAN, H B, *Jour Biol Chem*, **58**: 395 (1923)
[2] STEPHENSON, M, "Bacterial Metabolism," Longmans, Green & Company, New York, 1930

not prove that it was an intermediate product. Even though acetaldehyde was not produced from pyruvic acid, this fact cannot be taken as proof that it does not arise from some other source.

Kluyver[1] and his associates have formulated a scheme for the butyric acid fermentation, which follows:

1. $C_6H_{12}O_6 \rightarrow 2C_3H_4O_3$

2. $CH_3 CO C\overset{H}{\underset{\underset{\text{Methylglyoxal}}{|}}{-}}OH \rightarrow CH_3 CHO + HCOOH$

   Methylglyoxal   Acetaldehyde   Formic
   hydrate   acid

3. $C\overset{H}{\underset{\underset{\text{Formic}}{||}}{-}}OH \rightarrow CO_2 + H_2$

   Formic   Carbon   Hydrogen
   acid   dioxide

4. $CH_3CHO + H_2O \rightleftharpoons CH_3 C\overset{H}{\underset{\underset{\text{Acetic acid}}{|}}{-}}OH \rightarrow CH_3COOH + H_2$

5. $CH_3C\overset{O}{\underset{\underset{\text{Acetaldehyde}}{H}}{\diagdown}} + H\overset{H}{\underset{\underset{\text{Acetaldehyde}}{H}}{-}}C CHO \rightarrow CH_3 CHOH CH_2 CHO \rightleftharpoons CH_3 CH=CH C\overset{H}{\underset{OH}{-}}OH$

   Acetaldehyde   Acetaldehyde   Acetaldol   ↓
                                                  $CH_3 CH_2CH_2COOH$
                                                      Butyric acid
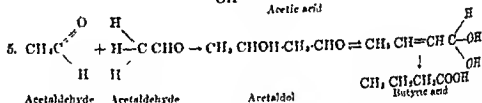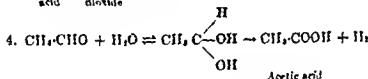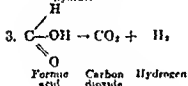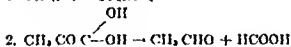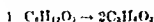
In the butyric acid fermentation, the main end products are butyric acid, acetic acid, carbon dioxide, hydrogen, and traces of formic acid

Kluyver is of the opinion that the mechanism for the conversion of sugar to hydrated methylglyoxal in the butyric acid fermentation is analogous to that of the alcoholic fermentation.

According to the scheme shown above, the weight of the carbon dioxide formed should be equivalent to approximately one-half of the weight of the glucose fermented. (Compare with the ethanol fermentation.) The number of molecules of acetaldehyde required for the formation of acetic acid and butyric acid should be equivalent to the number of molecules of carbon dioxide produced. Also the total number of molecules of hydrogen gas evolved (Eqs. 3 and 4) should be greater than the number of molecules of acetic acid produced (Eq. 4) by the number of molecules of carbon dioxide evolved. The following table indicates that these requirements are reasonably satisfied.

[1] KLUYVER, A. J., "The Chemical Activities of Micro-organisms," University of London Press, Ltd , 1931.

TABLE 73.—FERMENTATION BALANCE OF GLUCOSE, USING *Clostridium saccharobutylicum*[1]

(Medium: yeast extract, 2 per cent glucose, 1 per cent calcium carbonate, 30°C.)

Products	Grams	Per cent of glucose fermented	No. of mols per 50 mols of glucose fermented		
			CO_2	H_2	Acetal-dehyde
Glucose added	39 45				
Glucose unfermented	3 10				
Glucose fermented	36 35				
Carbon dioxide	17 4	47 8	97 8		
Hydrogen	0 94	2 59		116 6	
Formic acid	Traces				
Acetic acid	3 17	14 2		−21 3	21 3
Butyric acid	13 4	36 0			75 5
Total			97 8	95 3	96 8

[1] KLUYVER, A. J., "The Chemical Activities of Micro-organisms," University of London Press, Ltd., 1931.

TABLE 74.—FERMENTATION BALANCE OF GLUCOSE, USING *Cl. acetobutylicum*[1]

(Medium yeast extract, 2 per cent glucose, 37°C.)

Products	Grams	Per cent of glucose fermented	Mols per 50 mols of glucose fermented		
			CO_2	H_2	Acetal-dehyde
Glucose added	11 78				
Glucose unfermented	0 41				
Glucose fermented	11 37				
Carbon dioxide	6 14	54 0	119 3		
Hydrogen	0 1770	1 5		70 2	
Formic acid	Traces				
Acetic acid	0 53	4 7		−7 0	7.0
Butyric acid	0 24	2 1			4 2
Ethyl alcohol	0 27	2 4		4 7	4 7
Butyl alcohol	2 62	23 0		56 0	56 0
Acetone	0 82	7 2	−11 2	−22 4	22 4
Acetylmethylcarbinol	0 35	3 1			6 4
Total			99 1	101 5	100.7

[1] VAN DER LEK, J. B., "Onderzoekingen over de Butylalkoholgisting," Delft, 1930.

SCHEME FOR THE BUTYL ALCOHOL FERMENTATION[1]

1. $C_6H_{12}O_6 \rightarrow 2C_3H_6O_3$ (by an internal mechanism corresponding
to that of the alcoholic fermentation)

2. $CH_3 \cdot CO \cdot \underset{\underset{H}{|}}{C} - OH \rightarrow CH_3CHO + HCOOH$

   Methylglyoxal   Acetaldehyde Formic acid
   hydrate

Dehydrogenation Reactions

3. $\underset{\underset{O}{\|}}{C} - OH \rightarrow CO_2 + 2H$

   Formic acid

4. $CH_3 \cdot CHO + H_2O \rightleftharpoons CH_3 \cdot \underset{\underset{OH}{|}}{\overset{\overset{H}{|}}{C}} - OH \rightarrow CH_3COOH + 2H$

   Acetaldehyde   Acetic acid

Condensation Reactions

5. $CH_3 \cdot \underset{\underset{H}{|}}{\overset{\overset{O}{\|}}{C}} + H - \underset{\underset{H}{|}}{\overset{\overset{H}{|}}{C}} \cdot CHO \rightarrow CH_3 \cdot CHOH \cdot CH_2 \cdot CHO \rightleftharpoons$

   Acetaldehyde   Acetaldehyde   Acetaldol

$CH_3 \cdot CH{=}CH \cdot \underset{\underset{OH}{|}}{C} - OH \rightarrow CH_3 \cdot CH_2 \cdot CH_2 \cdot COOH.$

   Crotonaldehyde hydrate   Butyric acid

6. $CH_3 \cdot \underset{\underset{OH}{|}}{\overset{\overset{O}{\|}}{C}} + H - \underset{\underset{H}{|}}{C} \cdot COOH \rightarrow CH_2 \cdot \underset{\underset{OH}{|}}{\overset{\overset{OH}{|}}{C}} \cdot CH_2COOH \rightleftharpoons H_2O +$

   Acetic acid   Acetic acid

$CH_3 \cdot CO \cdot CH_2 \cdot \underset{\underset{OH}{|}}{\overset{\overset{O}{\|}}{C}} \rightarrow CH_3 \cdot CO \cdot CH_3 + CO_2$

   Acetyl acetic acid   Acetone   Carbon
   (Acetoacetic acid)   dioxide

Hydrogenation Reactions

7. $2H \rightarrow H_2$

8. $CH_3 \cdot CHO + 2H \rightarrow CH_3 \cdot CH_2OH$
   Acetaldehyde   Ethyl alcohol

9. $CH_3 \cdot CH_2 \cdot CH_2 \cdot COOH + 4H \rightarrow CH_3 \cdot CH_2 \cdot CH_2 \cdot CH_2OH + H_2O$
   Butyric acid   Butyl alcohol

10. $CH_3 \cdot CO \cdot CH_3 + 2H \rightarrow CH_3 \cdot CHOH \cdot CH_3$
   Acetone   Isopropyl alcohol

[1] KLUYVER, A J., "The Chemical Activities of Micro-organisms," University of London Press, Ltd.,
1931.

VAN DER LEE, J. R., "Onderzoekingen over de Butylalkoholgisting," Naamlooze Vennootschap
W. D Meinema, Delft, 1930.

It is necessary to account for several additional products when one turns from the butyric acid to the butanol-acetone fermentation. A scheme for this fermentation, to be acceptable, must satisfy conditions in respect to the relative proportions of each of the nine or more products found in the breakdown of a sugar. The scheme suggested by Kluyver does this well according to the results of Tables 74 and 75, one of which gives data for a medium containing 2 per cent calcium carbonate.

TABLE 75.—FERMENTATION BALANCE OF GLUCOSE, USING Cl acetobutylicum[1]
(Medium: yeast extract, 2 per cent glucose, 2 per cent calcium carbonate, 37°C.)

Products	Grams	Per cent of glucose fermented	Mols per 50 mols of glucose fermented		
			CO_2	H_2	Acetaldehyde
Glucose added	11 68				
Glucose unfermented	0 43				
Glucose fermented	11 25				
Carbon dioxide	5 31	47 5	97 0		
Hydrogen	0 2344	2 08		93 8	
Formic acid	Traces				
Acetic acid	1 15	10 2		−15.4	15 4
Butyric acid	2 97	26 6		. . . . . .	61 8
Ethyl alcohol	0 39	3 5		6 8	6 8
Butyl alcohol	0 48	4 3		10 2	10 2
Acetone	Traces				
Acetylmethylcarbinol	0 28	2 5			5 2
Total .			97 0	95 4	99 4

[1] VAN DER LEK, J. B., "Onderzoekingen over de Butylalkoholgisting," Delft, 1930.

Studies with Heavy Carbon Acetic and Butyric Acids and Acetone.—Wood, Brown, and Werkman (1945) studied the mechanism of the butanol fermentation by Cl. acetobutylicum and Cl. butylicum, using heavy carbon acetic and butyric acids and acetone. When heavy carbon acetic acid ($CH_3 \cdot C^{13}OOH$) was added to corn mash fermentations, the heavy carbon (C^{13}) was recovered in butanol, acetone, isopropyl alcohol, butyric and acetic acids, ethanol, and carbon dioxide.

The butanol contained about 50 per cent of it, which was present in about equal amounts in the carbinol and β-positions. Wood and his associates suggested that butanol was synthesized from acetic acid or a derivative of it.

The acetone and isopropyl alcohol contained from 15 to 19 per cent of the added heavy carbon atom, which was present in the carbonyl and

carbinol positions, respectively The heavy carbon recovery was almost the same in respect to these solvents. These facts were in accord with the suggestion that acetone is formed by the decarboxylation of aceto-acetic acid, which is produced from acetate.

When heavy carbon butyric acid $(CH_2 \cdot C^{13}H_2 \cdot CH_2 \cdot C^{13}OOH)$ was added to the medium, the heavy carbon (C^{13}) was recovered in butanol, ethanol, isopropyl alcohol, and acetic and butyric acids. About 85 per cent of the added heavy carbon was found in the carbinol and β-positions of the butanol, suggesting that butyric acid was the precursor of this alcohol. It was suggested by Wood and his coworkers that the conversion of butyric acid to acetic acid, acetone, and isopropyl alcohol may represent "a reversible series of reactions through acetoacetic acid"

When heavy carbon acetone $(CH_3 \cdot C^{13}OCH_3)$ was added to the mash, the C^{13} was recovered in the isopropyl alcohol. There was evidence that acetone was an intermediate in the formation of isopropyl alcohol.

References

ARZBERGER, C. F., W. H. PETERSON, and E. B. FRED: Certain Factors That Influence Acetone Production by *Bacillus acetoethylicum*, *Jour. Biol. Chem*, 44: 465-479 (1920)

BAKONYI, S : Versuche zur Theorie der Aceton-Ethylalkoholischen Gärung, *Biochem Zeit*, 169: 125 (1926).

BANZON, J R : Fermentative Utilization of Cassava, *Iowa State Coll. Jour. Sci*, 16: 15-18 (1941).

BEIJERINCK, M. W : Über die Butylalkoholgährung und das Butyl-ferment, *Verhandel Akad. Wetenschappen Amsterdam, Afdeel. Natuurkunde, Sectie II, Deel I,* 1893

——— : Sur la fermentation et le ferment butyliques, *Arch. néerland*, 29: 1-68 (1896)

——— : Sur les bacteries actives dans le Rousssage du Lin, *Arch néerland, Sci, Ser II*, 9 : 436-441 (1904).

BEHRENS, J : Untersuchungen über die Gewinnung der Hanffaser durch naturliche Rostmethoden, *Centr Bakt Parasitenk*, Abt II, 8: 114 (1902).

"Bergey's Manual of Determinative Bacteriology," 6th ed, The Williams & Wilkins Company, Baltimore, 1948

BERNHAUER, K, und K. KÜRSCHNER: Butyl—und Aceton—Garungen. I. Mitteilung Über Zwischenprodukte der Butanol-Aceton-Garung, *Biochem Zeit*, 280: 379-387 (1935).

BERTHELOT, A, et E. OSSART. Recherches sur les microbes producteurs d'acetone, *Compt rend.*, 173: 792-794 (1921)

BREDEMANN, G.: *Bacillus amylobacter* A. M. et Bredemann, *Centr Bakt Parasitenk*, Abt. II, 23: 385-568 (1909)

BOTKIN, S. Über einen *Bacillus Butyricus*, *Zeit. Hyg. Infectionskrankh*, 11: 421-434 (1892)

BROWN, R. W, H G WOOD, and C H. WERKMAN: Growth Factors for the Butyl Alcohol Bacteria, *Jour Bact*, 26: 206 (1938).

BRYNER, L. C., L M. CHRISTENSEN, and E I. FULMER: Hydrolysis of Oat Hulls with Hydrochloric Acid, *Ind Eng Chem*, 28: 206-208 (1936)

BUCHNER, E : Hoppe-Seyler Über den Einfluss des Sauerstoffs auf Gahrungen, *Zeit physiol Chem* , 9 : 380–415 (1885)

—— und J MEISENHEIMER: Über Buttersäuregärung, *Ber* , 41 : 1410–1419 (1908).

CHRISTENSEN, L M , and E I FULMER Analysis of n-Butanol, Acetone, and Ethanol in Aqueous Solution, *Ind Eng Chem (Anal Ed)*, 7: 180–182 (1935)

CHRISTENSEN, W B , M J JOHNSON, and H PETERSON Properties of the Lactic Acid-racemizing Enzyme of *Clostridium butylicum*, *Jour Biol Chem* , 127: 431 (1939)

DAVIES, R · Studies in the Acetone-Butyl Alcohol Fermentation 2 Intermediates in the Fermentation of Glucose by *Cl acetobutylicum*, 3 Potassium as an Essential Factor in the Fermentation of Maize Meal by *Cl acetobutylicum* (BY), *Biochem Jour* , 36 : 582–599 (1942)

—— . Studies on the Acetone-Butanol Fermentation 4 Acetoacetic Acid Decarboxylase of *Cl acetobutylicum* (BY), *Biochem Jour* , 37: 230–238 (1943)

—— and M STEPHENSON. Studies on the Acetone-Butyl Alcohol Fermentation 1 Nutritional and Other Factors Involved in the Preparation of Active Suspensions of *Cl acetobutylicum* (Weizmann), *Biochem Jour* , 35: 1320–1331 (1941)

DUCLAUX, E Sur la nutrition intracellulaire, *Ann Inst Pasteur* 9: 811–839 (1895)

DUNN, C G : "A Biochemical Investigation of the Metabolic Activities of *Bacillus Technicus*," Thesis (Ph D) , Department of Biology and Public Health, Massachusetts Institute of Technology, Cambridge, 1934

EMMERLING, O : Butylalkoholische Gärung, *Ber* 30 : 451–453 (1897)

FITZ, A *Bacillus butylicus*, *Ber* , 15 : 867–880 (1882)

—— : Über Spaltpilzgärungen, *Ber* , 17 : 1188 (1884)

FOWLER, G J , Y D WAD, and A G GOKHALE Acetone Fermentation Process in India, *Jour Indian Inst Sci* , 4 : 1–15 (1921)

—— and V SUBRAMANYAN Studies Relating to the Acetone Producing Organisms, *Jour Indian Inst Sci* , 8A : 71–87 (1925)

FRED, E B , W H PETERSON, and J A ANDERSON· Production of Acetone, Alcohol and Acids from Oat and Peanut Hulls, *Ind Eng Chem* , 15: 126 (1923)

——, —— , and W R CARROL The Occurrence of a Red Pigment Producing Organism in Corn Mash of the Acetone Butyl Alcohol Fermentation, *Jour Bact* , 10: 97–104 (1925)

——, —— , and M MULVANIA The Effect of Lactic Acid Bacteria on the Acetone-Butyl Alcohol Fermentation, *Jour Bact* , 11: 323–343 (1926)

FRIEBERG, G W : Observation on the Carbohydrate Metabolism of Acetone Butyl-Alcohol Fermentations, *Proc Soc Expt Biol Med* , 23 : 72–73 (1925)

FULTON, H L , W H PETERSON, and E B FRED· The Hydrolysis of Native Proteins by *B granulobacter pectinovorum* and the Influence of the Carbohydrate-protein Ratio on the Products of Fermentation, *Centr Bakt Parasitenk* Abt II, 27: 1–11 (1926)

GABRIEL, C L : Butanol Fermentation Process, *Ind Eng Chem* , 20: 1063–1067 (1928)

—— and F M CRAWFORD· Development of the Butyl-acetone Fermentation Industry, *Ind Eng Chem* , 22: 1163–1165 (1930)

GILL, A : The Acetone Fermentation Process and Its Technical Application, *Jour Soc Chem Ind* , 38: 273–282T (1919)

—— · The Production of Normal Butyl Alcohol and Acetone by Fermentation of Horse Chestnuts, *Jour Soc Chem Ind* , 38: 411–412T (1919)

GRASSBERGER, R , and A SCHATTENFROH: Über Buttersäuregärung, *Arch Hyg* , 42: 219–216 (1902)

GRIMBERT, M. L.: Fermentation anaerobie produite par le *Bacillus orthobutylicus*, *Ann. Inst. Pasteur*, 7: 353–402 (1893).

GRUBER, M.: Eine Methode der Cultur anaerobischer Bacterien Bemerkungen zu nebst Morphologie der Buttersäuregärung, *Centr. Bakt. Infektionskrankh*, 1: 367–372 (1887).

HIGGINS, G. A.: Solvents from Kelp, *Ind. Eng., Chem*, 10: 858–859 (1918).

KENTNER, J: Über das Vorkommen und die Verbreitung stickstoffbindender Bakterien in Meere, *Wiss Meeresuntersuch.*, N. F. 8, p. 42 (1904).

KILLEFFER, D. H.: Butanol and Acetone from Corn, *Ind. Eng. Chem.*, 19: 46–50 (1927).

KLUYVER, A J.: "The Chemical Activities of Micro-organisms," University of London Press, Lt , London, 1931.

———: Die bakteriellen Zuckervergährungen, *Ergeb. Enzymforsch*, 4: 230–273 (1935)

KNAYSI, G , and S. R DUTKY: *The Growth of a Butanol Clostridium in Relation to the Oxidation-reduction Potential and Oxygen Content of the Medium*, *Jour. Bact* , 31: 137–149 (1936).

LANGLYKKE, A F , and E. B. FRED: Products from the Fermentation of Glucose and Arabinose by Butyric Acid Anaerobes, *Jour. Bact.*, 29: 86–87 (1935).

———, W H PETERSON, and E. B FRED: Reductive Processes of *Clostridium butylicum* and the Mechanism of Formation of Isopropyl Alcohol, *Jour. Bact*, 34: 443–453 (1937).

———, and E McCOY: Products from the Fermentation of Glucose and Arabinose by Butyric Acid Anaerobes, *Jour. Bact.*, 29: 333–347 (1935).

———, J. M. VAN LANEN, and D. R. FRASER: Butyl Alcohol from Xylose Saccharification Liquors from Corncobs, *Ind. Eng. Chem.*, 40 (No. 9): 1716–1719 (1948)

LEONARD, R. H., W. H PETERSON, and G. J. RITTER: Butanol-Acetone Fermentation of Wood Sugar, *Ind Eng Chem*, 39: 1443–1445 (1947).

MARVEL, C S , and A E BRODERICK: The Composition of Yellow Oil Obtained in the Manufacture of n-Butyl Alcohol by Fermentation, *Jour. Am. Chem. Soc*, 47: 3045–3051 (1925).

McCLUNG, L S , E McCoy, and E. B FRED: Further Extensive Uses of the Vegetative Tissue Anaerobic System, *Centr. Bakt. Parasitenk.*, Abt II, 91: 225–227 (1935).

McCoy, E., E B FRED, W H PETERSON, and E G HASTINGS: A Cultural Study of the Acetone Butyl Alcohol Organism, *Jour. Infectious Diseases*, 39: 457–483 (1926).

———, ———, ———, and ———: A Cultural Study of Certain Anaerobic Butyric-acid Forming Bacteria. *Jour. Infectious Diseases*, 46: 118–137 (1930).

——— and L S McCLUNG; The Serological Agglutination of *Clostridium acetobutylicum* and Related Species, *Jour. Infectious Diseases*, 56: 333–346 (1935).

McDANIEL, L E., D. W. WOOLLEY, and W. H. PETERSON: Growth Factors for Bacteria VII Nutritional Requirements of Certain Butyl-alcohol Producing Bacteria, *Jour. Bact*, 37: 259 (1939).

MORIKAWA, K : "A New Butyl and Isopropyl Alcohol Fermentation," Thesis (Ph.D), Department of Biology and Public Health, Massachusetts Institute of Technology, Cambridge, 1925.

——— and S. C PRESCOTT: A New Fermentation Yielding Butyl and Isopropyl Alcohols, *Jour Bact*, 13: 58 (1928).

NATHAN, F : The Manufacture of Acetone, *Jour. Soc. Chem. Ind.*, 38: 271–273T (1919)

NORD, E F.: Chemical Processes in Fermentations, *Chem Rev.*, 3: 41–79 (1926).

NORTHROP, J. H., L. H. ASHE, and J. K. SENIOR: Biochemistry of *Bacillus Aceto-ethylicum* with Reference to the Formation of Acetone, *Jour. Biol. Chem.*, **39**: 1–21 (1919).

O-BURN, O. L.: The Production of Butyl and Isopropyl Alcohols by Fermentative Processes, *Iowa State College Jour. Sci.*, **10**: 97–98 (1935).

PASTEUR, L.: Extrait des procès-verbaux, *Soc. Chim. Paris* (Bull. p. 52) (1862).

PERDRIX, M. L.: Sur les fermentations produites par un microbe anaerobie de l'eau, *Ann. Inst. Pasteur*, **5**: 287–311 (1891).

PETERSON, W. H., and E. B. FRED: Butyl-acetone Fermentation of Corn Meal, *Ind. Eng. Chem.*, **24**: 237 (1932).

———, and B. P. DOMOGALLA: The Proteolytic Action of *Bacillus granulo-bacter pectinovorum* and Its Effect on the Hydrogen-ion Concentration, *Jour. Am. Chem. Soc.*, **46**: 2086–2090 (1924).

———, ———, and E. G. SCHMIDT: The Fermentation of Pentoses by *Bacillus granulobacter pectinovorum*, *Jour. Biol. Chem.*, **60**: 627–631 (1924).

———, and J. H. VERHULST: A Fermentation Process for the Production of Acetone, Alcohol, and Volatile Acids from Corncobs, *Ind. Eng. Chem.*, **13**: 757–759 (1921).

——— and M. J. JOHNSON: Fermentation of Acetoacetic Acid and Pyruvic Acid by the Acetone-Butanol Organism, *Clostridium acetobutylicum*, *Jour. Bact.*, **25**: 69 (1933).

PRAŻMOWSKI, A.: Zur Entwickelungsgeschichte und Fermentwirkung einiger Bac-terien-Arten, *Botan. Zeit.*, **37**: 409–422 (1879).

PRESCOTT, S. C., and K. MORIKAWA: Production of Butyl and Isopropyl Alcohols, U.S. Pat. 1,933,683.

PRINGSHEIM, H.: Über ein Stickstoff assimilierendes *Clostridium*, *Centr. Bakt. Para-sitenk.*, Abt. II, **16**: 795–800 (1906).

REILLY, J., and W. J. HICKINBOTTOM: Mechanism of the "*n*-Butyl Alcohol and Acetone" Fermentation Process, *Chem. News*, **119**: 169–170 (1919).

———, ———, F. R. HENLEY, and A. C. THAYSEN: The Products of the "Acetone: *n*-Butyl Alcohol" Fermentation of Carbohydrate Material with Special Refer-ence to Some of the Intermediate Substances Produced, *Biochem. Jour.*, **14**: 229–251 (1920).

REINKE, J., und G. BERTHOLD: Die Zersetzung der Kartoffel durch Pilze, *Untersuch. botan. lab. Univ. Göttingen*, p. 1 (1879).

ROBINSON, G. C.: A Study of the Acetone and Butyl Alcohol Fermentation of Various Carbohydrates, *Jour. Biol. Chem.*, **53**: 125–154 (1922).

SCHARDINGER, F.: *Bacillus macerans*, ein Aceton bildender Rottebacillus, *Centr. Bakt. Parasitenk.*, Abt. II, **14**: 772–781 (1905).

———: Verhalten von Weizen und Roggenmehl zu Methylenblau und zu Stärke-kleister, *Centr. Bakt. Parasitenk.*, Abt. II, **18**: 748–767 (1907).

SCHOEN, M.: "The Problem of Fermentation," Chapman & Hall, Ltd., London, 1928.

SJOLANDER, N. O.: The Fermentation Products of *Clostridium thermosaccharolyticum*, *Jour. Bact.*, **34**: 419–428 (1937).

——— and E. McCOY: A Cultural Study of Some "Butyric" Anaerobes Previously Described in the Literature, *Centr. Bakt. Parasitenk.*, Abt. II, **97**: 314–324 (1937).

———, A. F. LANGLYKKE, and W. H. PETERSON: Butyl Alcohol Fermentation of Wood Sugar, *Ind. Eng. Chem.*, **30**: 1251 (1938).

SMITH, H. F., and W. L. OBOLD: "Industrial Microbiology," The Williams & Wilkins Company, Baltimore, 1930.

SPEAKMAN, H. B.: The Production of Acetone and Butyl Alcohol by a Bacteriologic Process, *Jour. Soc. Chem. Ind.*, **38**: 155–161T (1919).

————: Seed-culture Methods in the Production of Acetone and Butyl Alcohol by Fermentation Process, *Ind. Eng. Chem.*, **12**: 581–587 (1920).

————: The Biochemistry of the Acetone and Butyl Alcohol Fermentation of Starch by *Bacillus granulobacter pectinovorum*, *Jour. Biol. Chem.*, **41**: 319–343 (1920)

————: Gas Production during the Acetone and Butyl Alcohol Fermentation of Starch, *Jour. Biol. Chem.*, **43**: 401–411 (1920).

————: Molecular Configuration in the Sugars and Acid Production by *Bacillus granulobacter pectinovorum*, *Jour. Biol. Chem.*, **58**: 395–413 (1923)

———— and J. F. PHILLIPS: A Study of a Bacterial Association, *Jour. Bact.*, **9**: 183–20 (1924)

————: The Biochemistry of Acetone Formation from Sugars by *Bacillus acetoethylicum*, *Jour. Biol. Chem.*, **64**: 41–52 (1925).

———— The Physiological Significance of Deamination in Relation to Glucose Oxidation, *Jour. Biol. Chem.*, **70**: 135–150 (1926).

STEPHENSON, MARJORY: "Bacterial Metabolism," Longmans, Green & Company, New York, 1930

STILES, H. R., W. H. PETERSON, E. B. FRED: The Nature of the Acids Produced in the Fermentation of Maize by *Clostridium acetobutylicum*, *Jour. Biol. Chem.*, **84**: 437–453 (1929).

STORMER, K.: Über die Wasserroste des Flachses, *Centr. Bakt. Parasitenk.*, Abt II **13**: 171 (1904).

TATUM, E. L., W. H. PETERSON, and E. B. FRED: An Unknown Factor Stimulating the Formation of Butyl Alcohol by Certain Butyric Acid Bacteria, *Jour. Bact.* **27**: 207–217 (1931)

————, ————, and ————: Identification of Asparagine as the Substance Stimulating the Production of Butyl Alcohol by Certain Bacteria, *Jour. Bact.*, **29**: 563–572 (1935)

THAYSEN, A. C.: Bacteriology of the Process for Acetone and Butyl Alcohol Manufacture, *Jour. Inst. Brewing*, **27**: 529–542 (1921).

UNDERKOFLER, L. A. L. M. CHRIST . . . . tonic Fermentation of Xylose and Other . . . .

————, E. I. FULMER, and M. M. RAYMAN: Oat Hull Utilization by . . . . tion, *Ind. Eng. Chem.*, **29**: 1290–1292 (1937).

———— and J. E. HUNTER, JR.: Butyl-acetonic Fermentation of Arabinose and Other Sugars, *Ind. Eng. Chem.*, **30**: 480 (1938).

VAN DER LEK, J. B.: "Onderzoekingen over de Butylalkoholgisting." Thesis, Technische Hoogeschool, Delft, 1930

VAN TIEGHEM, M.: Sur le *Bacillus amylobacter* et son rôle dans la putréfaction des tissues végétaux, *Bull. soc. botan. France*, **24**: 128–135 (1877)

————: Identité du *Bacillus amylobacter* et du vibrion butyrique de M. Pasteur, *Compt rend*, **89**: 5–8 (1879)

VON KLECKI, V.: Ein neuer Buttersäuregärungserreger, *Centr. Bakt. Parasitenk.*, Abt II, **2**: 169–184 (1896).

WAKSMAN, S., and D. KINSH: Butyric Acid and Butyl Alcohol Fermentation of Hemicellulose- and Starch-rich Materials, *Ind. Eng. Chem.*, **25**: 1036–1041 (1933)

WEBER, H. H., and W. KOCH: Zur Methodik der Analyse technischer Losungsmittel, *Chem. Ztg.*, **57**: 73–74 (1933)

WEINSTEIN, L., and L. F. RETTGER: Some Factors Involved in the Biological Production of Acetone and Butyl Alcohol, *Jour. Bact.*, **23**: 74–75 (1932)

WENDLAND, R T., E I. FULMER, and L. A. UNDERKOFLER: Butyl-Acetonic Fermentation of Jerusalem Artichokes, *Ind. Eng. Chem.*, **33**: 1078–1081 (1941).

WEYER, E. R., and L. F. RETTGER: A Comparative Study of Six Different Strains of the Organism Commonly Concerned in Large-scale Production of Butyl Alcohol and Acetone by the Biological Process, *Jour Bact*, **14**: 399–424 (1927).

WILEY, A. J , M. J JOHNSON, E. MCCOY, and W. H PETERSON: Acetone-Butyl Alcohol Fermentation of Waste Sulfite Liquor, *Ind. Eng Cchm*, **33**: 606–610 (1941).

WILSON, P W , W H PETERSON, and E. B. FRED: The Production of Acetylmethyl Carbinol by *Clostridium acetobutylicus*, *Jour. Biol. Chem.*, **74**: 495–507 (1927).

————, ————, and ————: The Relationship between the Nitrogen and Carbon Metabolism of *Clostridium acetobutylicum*, *Jour Bact.*, **19**: 231–260 (1930).

WINOGRADSKY, S : *Clostridium pastorianum*, seine Morphologie und seine Eigenschfter als Buttersäurefermert, *Centr Bakt. Parasitenk*, Abt. II, **9**: 43–54, 107–112 (1902)

WOOD, H. G , R. W BROWN, and C H. WERKMAN: Mechanism of the Butyl Alcohol Fermentation with Heavy Carbon Acetic and Butyric Acids and Acetone, *Arch Biochem*, **6**: 243–260 (1945).

WYNKOOP, R : n-Butanol and Acetone, *Ind Eng Chem*, **35**: 1240–1242 (1943)

WYNNE, A M : Inhibition of the Acetone-butyl Alcohol Fermentation by Acids, *Jour Bact*, **22**: 209–237 (1931)

Some Patents

ARZBERGER, C F Process for the Production of Butyl Alcohol by Fermentation, U S Patent 2,050,219, Aug 4, 1936

BAYER & Co . Production of Acetone and Alcohol by Fermentation of Sugar and Saccharine Materials by *Bacillus Macerans*, German Patent 283107, 1013

BOINOT, F Manufacture of Normal Butyl Alcohol, U.S. Patent 1,565,513, Dec 15, 1925

DESBOROUGH, A P H., A C THAYSEN, J REILLY, and F R HENLEY: The Manufacture of Acetone and Other Products by Fermentation, British Patent 128,714, 1919

EDMONDS, W J Butyl Acetonic Fermentation Process, U S. Patent 1,740,162, Dec. 17, 1929

FERNBACH, A Acetono-butylic Fermentation Process, U.S Patent 1,854,895, Apr. 19, 1932

———— and E H STRANGE Acetone and Higher Alcohols (Amyl, Butyl or Ethyl Alcohols and Butyric, Propionic or Acetic Acid) from Starches, Sugars and Other Carbohydrates, British Patents 15,203–15,201 1911

———— and ———— Fermentation Process for the Production of Acetone and Higher Alcohols from Starch, Sugars and Other Carbohydrate Material, U S Patent 1,044,368, Nov 12 1912

———— and ———— Manufacture and Production of a Nutrient Medium for Use in Fermentation Processes, U S Patents 1,044,416–1,044,417, Nov 12, 1912.

———— and ———— Production of Acetone, Acetates and Pyruvates, British Patent 11,607, 1915

FRILING, G W.: Process for Producing Acetone and Butyl Alcohol, U.S Patent 1,537,597, May 12, 1925

———— Fermentation Process for Production of Acetone and Butyl Alcohol, U.S Patent 1,551,550, Sept 1, 1925

————. Process for Making Acetone and Butyl Alcohol, U S Patent 1,672,487, June 5, 1928

FREY, A., and H. GLÜCK: Process of Producing Butyl Alcohol and Acetone by Fermentation, U.S. Patent 2,146,892, Feb. 14, 1939.

———, ———, and H. OEHME: Production of Butanol and Acetone by the Fermentation of Whey, U.S. Patent 2,166,047, July 11, 1939.

FUNK, F. J.: Butyl Alcohol and Acetone Fermentation Process, U.S. Patent 1,538,516, Mar. 19, 1925.

GERRETSEN, F C : Biological Production of Butyl Alcohol and Acetone, U.S. Patent 1,858,808, May 17, 1932.

HALL, H E.: Fermentation Process for Solvent Manufacture, U.S. Patent 2,147,487, Feb. 14, 1939.

HANER, C, MOYLAN, and O. GAMPER: Process for Producing Butyl Alcohol and Acetone, and Ethyl Alcohol, U.S. Patent 1,885,096, Oct. 25, 1932

HILDEBRANDT, F M., and N. M. ERB: Fermentation Process, U.S. Patent 2,169,244, Aug 15, 1939.

——— and ———: Butyl Acetone Fermentation Process, U.S. Patent 2,169,246, Aug. 15, 1939

HORTON, G D : Process of Producing Butyl Alcohol and Acetone by Fermentation, U S. Patent 1,427,595, Aug 20, 1922.

HUTCHINSON, H B : Production of Butyl Alcohol and Acetone by Fermentation, U.S. Patent 1,898,961, Feb 21, 1933.

———: Production of Butyl Alcohol and Acetone by Fermentation, U.S. Patent 1,928,379, Sept. 26, 1933.

IZSÁK, A : Process for Producing Butyl Alcohol and Acetone, U.S Patent 2,016,112, Oct 1, 1935

——— and F. J. FUNK: Process of Producing Butyl Alcohol, U S. Patent 1,908,361, May 9, 1933.

LEGG, D. A · Process for the Production of Butyl Alcohol and Acetone, U.S. Patent 1,582,408, Apr. 27, 1926

———: Art of Butyl-acetonic Fermentation, U.S. Patent 1,668,814, May 8, 1928

———: Process of Obtaining Butyl Alcohol and Acetone by Fermentation, U S Patent 1,927,814, Sept 19, 1923.

———: Production of Butyl Alcohol by Fermentation, U.S. Patent 2,023,368, Dec 3, 1935

———: Production of Ethyl Alcohol and Acetone by Fermentation, U S Patent 2,036,450, Dec. 8, 1936

——— and L. M CHRISTENSEN: Production of Butyl Alcohol and Acetone by Fermentation, U S. Patent 1,913,164, June 6, 1933

——— and H. R STILES· Process of Producing Butyl Alcohol, U.S Patent 2,089,562, Aug. 10, 1937

——— and M. T. WALTON: Butyl Alcohol Fermentation, U S Patent 2,132,358, Oct 4, 1938.

LIEBESNY, P, and H WERTHEIM: Method of Promoting the Growth and Activity of Microorganisms, U.S. Patent 2,133,203, Oct. 11, 1938.

LOUGHLIN, J F.: Production of Butyl Alcohol and Acetone by Fermentation, U S Patent 1,992,921, Feb. 26, 1935.

———: Manufacture of Butyl Alcohol, Acetone and Isopropyl Alcohol, U.S. Patent 2,096,377, Oct. 19, 1937.

McCoy, E F.: Production of Butyl Alcohol and Acetone by Fermentation, U S. Patent 2,110,109, Mar. 1, 1938.

McCUTCHAN, W. N., and C F ARZBERGER. Production of Butyl Alcohol by Fermentation, U S Patent 2,023,087, Dec 3, 1935

MULLER, J.: Production of Neutral Solvents by Fermentation, U S Patent 2,132,039, Oct. 4, 1938

NORTHROP, J. H. Process for the Production of Acetone, U S Patent 1,293,172, Feb. 4, 1919.

PIKE, E F., and H. F. SMITH: Process of Producing Acetone and Butyl Alcohol by Fermentation, U S. Patent 1,655,435, Jan 10, 1928

RICARD, E : Manufacture of Acetone, U S Patent 1,385,888, July 26, 1921

――――: Manufacture of Acetone and Butyl Alcohol by Fermentation, U S Patent 1,550,746, Aug 25, 1925.

――――, ALLENET et CIE Manufacture of Acetone and Butyl Alcohol by Fermentation, British Patent 176,284, Mar. 9, 1922

ROBINSON, G C. Production of Butyl Alcohol and Acetone by the Fermentation of Molasses, U S Patent 1,510,526, Oct. 7, 1924.

SCHECKENBACH, J · Process of Manufacturing Fused Oil, U S Patent 1,118,238, Nov. 24, 1914.

STILES, H R. Butyl-acetonic Fermentation Process, U.S Patent 2,023,374, Dec 3, 1935.

―――― Butyl-acetonic Fermentation Process, U S Patent 2,098,200, Nov 2, 1037

STRANGE, E H . Process for the Manufacture of Alcohols and Acetone, U S Patent 1,550,928, Aug 25, 1925

WATERS, L. W.· Fermentation Process, U S Patent 1,546,694, July 21, 1925

WEIZMANN, C.. Production of Acetone and Alcohol by Bacteriological Processes, U S. Patent 1,315,585, Sept 9, 1919

―――― and H M SPIERS· Fermentation of Starchy Material for the Production of Acetone and Butyl Alcohol, British Patent 164,762, Jan 2, 1919

―――― and G A HAMLYN. Fermentation Process for the Production of Acetone and Butyl Alcohol, U S Patent 1,329,214, Jan 27, 1920

――――: Fermentation Process for the Production of Acetone and Butyl Alcohol, U S Patent 1,437,697, Dec 5, 1922

――――. Acetone and Butyl Alcohol, British Patent 496,137, Nov. 24, 1938.

――――: Production of Acetone and Butyl Alcohol from Molasses, French Patent 838,258, Mar 2, 1939

WERTHEIM, H · Process for the Simultaneous Production of Butyl Alcohol and Acetone by Fermentation, U S Patent 1,917,676, July 11, 1933

――――: Process for Producing Butyl Alcohol and Acetone by Fermentation, U S. Patent 1,917,677, July 11, 1933.

WEYER, E R.: Fermentation Process, U S Patent 1,696,022, Dec 18, 1928

WHEELER, M C., and C D. GOODALE. Continuous Butyl Alcohol Fermentation Process. U S Patent 1,875,536, Sept 6. 1932

WOODRUFF, J C, H R STILES, and D A LEGG Process of Producing Butyl Alcohol, U.S. Patent 2,089,522, Aug 10, 1937.

THE ACETONE-ETHANOL FERMENTATION

The acetone-ethanol fermentation differs from the acetone-butanol fermentation in several respects, namely, the type of organism used, the end products, in particular with respect to the quantities of butyric acid and butanol formed; the optimum pH; the use of calcium carbonate, and the time required for the completion of the fermentation.

TABLE 76.—QUANTITIES OF ACETONE AND ALCOHOL PRODUCED BY B. acetoethylicus FROM VARIOUS SUBSTRATES[1]

(Medium, 2 per cent sugar, 0 5 per cent peptone, and 2 per cent CaCO₃, temperature, 37°C.; incubation period, 10 days)

Substance	Acetone, per cent	Alcohol, per cent	Substance	Acetone, per cent	Alcohol, per cent
Galactose	4– 5	22–24	Dextrin.	6– 7	14–16
Maltose	6– 7	23–24	Dextrose..	9–10	22–23
Mannose.	6– 7	22–23	Levulose.	8–10	24–25
Raffinose	8–10	22–23	Xylose.	4– 5	18–20
d-Arabinose. .	6– 7	12–16	Glycerol	. . . . .	40–43
Calcium lactate. .	. . .	. . . . .	Sucrose.	8– 9	24–26
Starch .	8–10	20–24			

[1] NORTHROP, J. H , L H ASHE, and J K SENIOR, Jour. Biol. Chem , 39:1 (1919).

Schardinger[1] is credited with being the first to discover acetone as a product of bacterial fermentation. The organism isolated and investigated by him was designated as Bacillus macerans Acetone, ethanol, acetic and formic acids were the principal end products obtained from the fermentation of potatoes or potato starch media containing peptone and calcium carbonate (see page 314).

At the suggestion of the Council of National Defense, Northrop undertook research in an attempt to develop acetone through a fermentation process. In 1919, Northrop, Ashe, and Senior[2] isolated an organism, B. acetoethylicum (B. acetoethylicus Northrop et al.),[3] which produced

[1] SCHARDINGER, F., Centr. Bakt Parasitenk., Abt. II, 14: 772 (1905)
[2] NORTHROP, J. H , L H. ASHE, and J K. SENIOR, Jour. Biol. Chem , 39:1 (1919).
[3] "Bergey's Manual of Determinative Bacteriology," 6th ed , The Williams & Wilkins Company, Baltimore, (1918)

acetone from starch. A careful study was made of the organism and the biochemistry of the fermentation

B. acetoethylicus, which is closely related, if not identical, to *B. macerans*,[1] was isolated from some old potatoes It is a motile, spore-forming, Gram-negative, facultative anaerobe. It grows well in a 2 per cent corn medium containing calcium carbonate. Its optimum reaction for growth is a pH of 8 to 9; for fermentation, a pH of 6 to 8. It has an optimum temperature of 40 to 43°C. Some of its spores will withstand boiling for at least 20 min. Ethyl, propyl, and butyl alcohols; acetone; and formic acid may be formed from suitable carbon-containing compounds Table 76 shows some results of some fermentations.

Raw Materials.—A large number of carbohydrate substances may be used for the fermentation Corn, potatoes, and molasses are substances
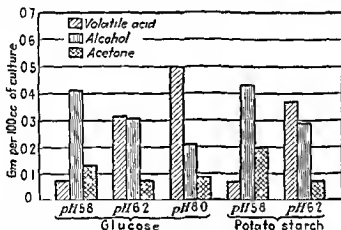
FIG. 61 —The influence of varying the reactions of the medium on the products of fermentation [*Courtesy of Arzberger, Peterson, and Fred, Jour. Biol. Chem.*, 44: 465 (1920)]

available in large quantities and at a reasonable cost. Hydrolyzed corncobs and oat and peanut hulls may serve as cheap sources of raw material. It is sometimes necessary to add peptone to a mash to supply the nitrogen required by the organism, as, for example, when the mash contains starch alone. The use of concentrations of about 2 to 3 per cent of carbohydrate substances is customary

pH.—According to Northrop and his associates,[2] the optimum pH range for the growth of *B acetoethylicus* (*B acetoethylicum*), was 8 to 9 However, highest yields were obtained when the fermentation mash was maintained at a pH of 6 to 8

In the fermentation of xylose syrup, obtained from the hydrolysis of

[1] *Ibid*

[2] NORTHROP, J H, L H ASHE, and R R MORGAN, *Jour. Ind. Eng. Chem*, 11: 723 (1919)

corncobs, Peterson and his associates[1] advised the use of an initial reaction of pH 7.6 to 8.4 and the provision of sufficient calcium carbonate to neutralize the acids as formed.

Arzberger, Peterson, and Fred[2] have shown that the reaction of the
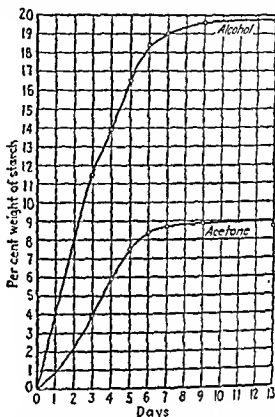
Fig. 62.—Rate of formation of acetone and alcohol.   [*Courtesy of Northrop, Ashe and Senior, Jour. Biol. Chem.,* 39: 1 (1919).]

medium plays an important part in determining the relative quantities of the various end products of the fermentation by *B. acetoethylicus.* An increase in the pH of the mash resulted in a decrease of alcohol production and an increase in the volatile acid content. A marked acid reaction, a pH of 5 8 to 6.0, favored the production of acetone and decreased the yield of acids. These results are indicated in Fig 61.

Calcium carbonate is always used in this type of fermentation to buffer the pH, in sharp contrast to the acetone-butanol fermentation. A 2 per cent concentration is satisfactory in most cases.

Optimum Temperature.—A temperature range of 40 to 43°C. is optimum for fermentations produced by *B. acetoethylicus.*

[1] Peterson, W. H , E. B. Fred, and J. H. Verhulst, *Ind. Eng Chem ,* 13: 757 (1921).

[2] Arzberger, C. F., W H. Peterson, and E. B. Fred, *Jour. Biol. Chem ,* 44: 465 (1920)

Duration of the Fermentation.—The fermentation ordinarily requires about 6 days. By using inert materials such as branches, coke, or corncobs to retain the slime which is formed during the fermentation and which contains bacteria and calcium carbonate, the time required for the fermentation may be considerably reduced. After the fermentation has been completed, the fermentation liquor is drawn off and a new mash added to the vat containing the slime-coated inert material. This process may be repeated several times, but it is necessary to use precautions to prevent contamination of the mash and to guard against sluggishness of the culture.

TABLE 77.—RATE OF FORMATION OF ACETONE AND ALCOHOL[1]
(Medium: 10 g of potato starch, 4 g of peptone, 10 g of calcium carbonate, and 500 cc. of water, temperature, 42°C, time analyzed as noted)

Time after inoculation, days	Culture No						Average		Ratio alcohol to acetone	
	1		2		3					
	Acetone, per cent	Alcohol, per cent	Acetone, per cent	Alcohol, per cent	Acetone, per cent	Alcohol, per cent	Acetone, per cent	Alcohol, per cent	By weight	By mols
3	4 6	12 3	4 0	11 4	3 3	10 7	3 9	11 5	2 9	3 65
4	7 4	16 1	5 6	13 5	4.6	12 3	5 9	13 9	2.3	2.9
5	8 9	18 1	7 4	16.6	6 1	14 7	7 5	16 5	2 2	2 8
6	9 2	18 9	8 6	18 4	7 4	18 0	8 4	18 4	2 2	2 8
7	9 3	19 4	9 0	19.4	7 7	18 1	8 7	18 9	2 2	2 8
9	9 3	19 5	9 1	19 4	8 3	20 0	8 9	19 6	2 2	2 8
13	9 4	19 5	9 0	19 3	8 8	20 2	8 7	19 6	2 2	2 8

[1] NORTHROP, J. H., L H ASHE, and J K SENIOR, Jour Biol Chem, 39:1 (1919).

The Fermentation of Corncobs.—Peterson, Fred, and Verhulst[1] (1921) devised a method for hydrolyzing corncobs and fermenting the sugars thus produced with Bacillus acetoethylicus. The corncobs were hydrolyzed for 1 hr. at a steam pressure of 20 lb. The ratio of the weights of the water, corncobs, and sulphuric acid used for the hydrolysis was 200:50:4. The hydrolyzate obtained was neutralized with calcium hydroxide and pressed, and the residue was washed. The sugar content of the mash was adjusted to a 3 per cent concentration (as glucose) The reaction of the fermentation was maintained between 7.6 and 8 4 at the beginning. Sufficient calcium carbonate was added to neutralize

[1] Loc cit

the acids formed. On the basis of 100 lb. of corncobs, the yield was 2.7 lb. of acetone, 6.8 lb. of ethanol, and 3.4 lb. of volatile acids.

The Fermentation of Oat and Peanut Hulls.—In 1923, Fred, Peterson, and Anderson[1] reported that *B. acetoethylicus* (*B. acetoethylicum*) produced acetone and ethanol from the sugars obtained by the hydrolysis of oat and peanut hulls. (These hulls are waste products obtained in the manufacture of oatmeal and peanut butter.) The hulls were hydrolyzed under a pressure of 15 lb. of steam for 2 hr. with 2 per cent sulphuric acid. The hydrolyzate was neutralized with milk of lime, and the sugars were extracted by pressing and washing. From oat hulls, as high as 26.5 per cent of reducing sugars was obtained as glucose; from peanut hulls, 7.6 per cent. The sugar concentration of the mash was adjusted to approximately 3 per cent as glucose; calcium carbonate, peptone, and sodium phosphate were added, and the mash was inoculated with *B acetoethylicus*. From 100 lb. of oat hulls, 7.2 lb. of ethanol, 3.9 lb of acetone, and 1.4 lb. of volatile acids were produced.

For further details of this fermentation consult some of the references listed at the end of Chap. XII.

Mechanism of the Ethanol-acetone Fermentation.—Neuberg and his associates suggested that acetone was produced by either the amylobacter or macerans type of organism according to the following sequence:

$$2CH_3 CHO \rightarrow CH_3 CHOH \cdot CH_2 \cdot CHO \rightarrow CH_3 \cdot CO \cdot CH_2 COOH \rightarrow CH_3 CO CH_3$$
$$\text{Acetaldehyde} \qquad \text{Aldol} \qquad \text{Acetoacetic acid} \qquad \text{Acetone}$$
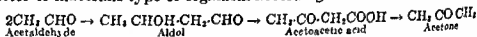
Speakman's Theory.—Speakman[2] contributed valuable additions to our knowledge of the biochemistry of the ethanol-acetone fermentation He demonstrated that hydrogen gas, as well as carbon dioxide gas, was formed during the fermentation of carbohydrates by *B. acetoethylicus* Previous workers had reported gas formation in terms of carbon dioxide only

Using data derived from his own experiments and those of previous investigators, Speakman formulated a scheme to show the mechanism of the formation of the end products of the fermentation. In the normal fermentation of *B acetoethylicus*, ethanol, but no acetone, is produced at the beginning of the fermentation; later both ethanol and acetone are formed simultaneously. Thus Speakman suggested that ethanol arose in two different ways: one involving a process in which no acetone was produced, the other associated with acetone formation.

Periodic neutralization of a fermenting mash with sodium hydroxide resulted in an increase in the volatile acid content and a decrease in the

[1] FRED, E. B., W. H. PETERSON, and J. A. ANDERSON, *Jour Ind Eng Chem*, **15**: 126 (1923)

[2] SPEAKMAN, H. B, *Jour. Biol. Chem.*, **64**: 41 (1925)

yields of ethanol and acetone. Hence, Speakman ruled out the possibility of a Cannizzaro reaction of the following nature.

$$CH_3 CHO + H_2 \rightarrow CH_3 CH_2OH$$
$$CH_3 CHO + O \rightarrow CH_3 COOH$$
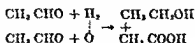
Speakman demonstrated experimentally that pyruvic acid was produced by *B. acetoethylicus* from both glucose and maltose during the first half of the fermentations. Later acetone accumulated, but free pyruvic acid could not be detected. These facts suggested that pyruvic acid might be an intermediate in the formation of acetone.

In the fermentation of glycerol there was an almost complete absence of both pyruvic acid and acetone. Speakman reasoned that acetone was not produced because its precursor, pyruvic acid, was not formed, except possibly in traces, from glycerol As an alternate explanation, he suggested that pyruvic acid, if produced in quantity, was converted to products other than acetone He added pyruvic acid, neutralized with sodium hydroxide, to mashes containing glycerol Acetone accumulated in the fermenting medium, part of it after the pyruvic acid had disappeared from the mash. Acetone was produced likewise from pure pyruvic acid. These facts indicated that pyruvic acid might be an intermediate product in the fermentation.

Since both formic and acetic acids were produced from pyruvic acid by fermentation with *B acetoethylicus*, Speakman suggested that they were formed from pyruvic acid according to the following equation:

$$CH_3 CO COOH + H_2O \rightarrow CH_3 COOH + HCOOH$$

Lactic acid was produced rapidly in the fermentation mash during the initial stages (Speakman). The production then fell to a constant level and continued thus to the end of the fermentation

Speakman proposed, as the result of the foregoing observations, the following scheme (1) for the production of substances during the first part of the fermentation:

I.
$$\begin{array}{c}
CH_3\ CHOH\text{-}COOH \\
\text{Lactic acid} \\
\uparrow + H_2 \\
C_6H_{12}O_6\ (-2H_2) \rightarrow CH_3\ CO\ COOH + H_2 \rightarrow CH_3\ CHO + CO_2 \\
\text{Glucose} \qquad\qquad \text{Pyruvic acid} \qquad\qquad \text{Acetaldehyde} \\
\downarrow + H_2O \qquad\qquad\qquad \downarrow + H_2 \\
CH_3\ COOH + H\ COOH \qquad CH_3\ CH_2OH \\
\text{Acetic acid} \quad \text{Formic acid} \qquad \text{Ethanol}
\end{array}$$
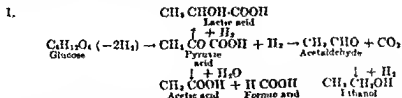
The following equations were suggested by Speakman to explain the changes that took place later in the fermentation, when acetaldehyde was found in the free state in the cell as the result of its more rapid formation than reduction:

$$2.\ 2CH_3 \cdot CHO \rightarrow \underset{\text{Aldol}}{CH_3 \cdot CHOH \cdot CH_2 \cdot CHO} \quad \textit{(Condensation reaction)}$$

$$\begin{cases} \underset{\text{Aldol}}{CH_3 \cdot CHOH \cdot CH_2 \cdot CHO} & O & \underset{\beta\text{-Hydroxybutyric acid}}{CH_3 \cdot CHOH \cdot CH_2 \cdot COOH} \\ & | & \rightarrow + \\ \underset{\text{Acetaldehyde}}{CH_3 \cdot CHO} & H_2 & \underset{\text{Ethanol}}{CH_3 \cdot CH_2OH} \end{cases} \begin{matrix}\textit{(Oxidation-} \\ \textit{reduction} \\ \textit{reactions)}\end{matrix}$$

$$\begin{cases} \underset{\substack{\beta\text{-Hydroxybutyric}\\ \text{acid}}}{CH_3 \cdot CHOH \cdot CH_2 \cdot COOH} & O & \underset{\text{Acetoacetic acid}}{CH_3 \cdot CO \cdot CH_2 \cdot COOH} + H_2O \\ & | & \rightarrow + \\ \underset{\text{Acetaldehyde}}{CH_3 \cdot CHO} & H_2 & \underset{\text{Ethanol}}{CH_3 \cdot CH_2OH} \end{cases}$$

$$\underset{\text{Acetoacetic acid}}{CH_3 \cdot CO \cdot CH_2COOH} \rightarrow \underset{\text{Acetone}}{CH_3 \cdot CO \cdot CH_3} + \underset{\substack{\text{Carbon} \\ \text{dioxide}}}{CO_2} \quad \begin{matrix}\textit{(Decarboxylation} \\ \textit{reaction)}\end{matrix}$$

In the fermentation of glycerol there was more active hydrogen available than was the case in the fermentation of glucose. Thus acetaldehyde was reduced principally to ethanol in the fermentation of glycerol, while only a slight amount was condensed to aldol. An increased production of acetaldehyde followed the addition of pyruvic acid to the glycerol fermentation, but there was no proportional increase in the production of active hydrogen. Accordingly, some of the acetaldehyde condensed to aldol, and the second part of the Speakman scheme became operative.

Bakonyi's Theory.—Bakonyi[1] doubted the theory that acetone was produced from aldol through β-hydroxybutyric acid, for he stated that neither *B. macerans* nor *B. acetoethylicus* fermented β-hydroxybutyric acid readily.

In the fermentations carried on by the acetone-ethanol organisms, Bakonyi (1926) observed that ethyl alcohol and acetone were produced from carbohydrates, in the presence of calcium carbonate, in the proportion of 2 molecules of the former to 1 molecule of the latter. The addition of acetaldehyde or aldol to a fermenting mash resulted in an increase of ethanol and acetone in the proportion of 2 molecules of ethanol to 1 molecule of acetone. From 2 molecules of acetic acid (calcium acetate), 1 molecule of acetone was produced.

On the basis of the foregoing data, Bakonyi suggested that aldol was dismutated to ethyl alcohol and acetic acid, the acetic acid yielding acetone. (Compare with the butanol-acetone fermentation.)

The fermentation of starch would be thus expressed as follows:

$$\underset{\text{Starch}}{2C_6H_{10}O_5} + 3H_2O \rightarrow \underset{\text{Ethanol}}{2CH_3 \cdot CH_2OH} + \underset{\text{Acetone}}{CH_3 \cdot CO \cdot CH_3} + 5CO_2 + 4H_2$$
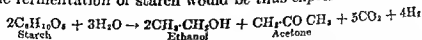
No matter what theory one accepts to explain the mechanism of the fermentation, it is necessary for the scheme selected to show bal-

[1] BAKONYI, S , *Biochem ZeiL*, **169**, 125 (1926).

anced oxidation-reduction relationships and carbon partition. Complex changes take place in the breakdown of protein materials. It is important not to forget that here too changes are taking place which have a strong influence on the types and quantities of the end products of the fermentation

References

See Chapter XII

CHAPTER XIV

THE BUTYL ALCOHOL–ISOPROPYL ALCOHOL FERMENTATION

The fermentation in which butyl alcohol and isopropyl alcohol are formed as the two principal products of commercial value is related in many respects to the acetone-butyl alcohol fermentation (Chap. XII) and the acetone-ethyl alcohol fermentation (Chap. XIII).

In fact, these three fermentations have many points of similarity both as to conditions that must be imposed for successful operations and as to the organisms involved. The bacteria causing these fermentations apparently all belong to the same group and are so nearly related that species differences are difficult to detect when they are described for classification purposes. In fermentations carried out on a sizable scale, however, the several species or strains show distinct differences in behavior and in the qualitative and quantitative analysis of the end products. Therefore it seems reasonable to regard them as separate fermentations.

Isopropyl alcohol (isopropanol) like butyl alcohol is a valuable solvent and can be used advantageously to replace ethyl alcohol or acetone in several industries, notably the manufacture of perfumes and toilet preparations.

The fermentation process may not become a very important one industrially since isopropyl alcohol is now obtained as a by-product in cracking petroleum in the manufacture of gasoline. The present demand is met in this way. As in the case of glycerol, the fermentation process may become economically advantageous under conditions of unusual demand.

Beijerinck[1] (1893) isolated an organism that produced butanol from malt sugar. This organism, although described as *Granulobacter butylicum*, has since been designated as *Clostridium butylicum* (Beijerinck) Donker.

The occurrence of isopropyl alcohol (CH_3,$CHOH$ CH_3) as a fermentation product was observed by Pringsheim[2] in 1906. The organism that gave rise to isopropyl alcohol was named *Cl. americanum* Pringsheim. This organism is now regarded as possibly identical with *Cl. butylicum* (Beijerinck) Donker.

[1] BEIJERINCK, M. W., *Verhandel. Akad. Wetenschappen Amsterdam, Afdel Naturkunde*, Qde Sectie II, No. 10 (1893).

[2] PRINGSHEIM, H. H., *Centr. Bakt. Parasitenk.*, Abt II, 16: 795 (1906).

Folpmers[1] (1920) isolated *Granulobacter butylicum* [*Cl. butylicum* (Beijerinck) Donker] from malt and demonstrated that the products of fermentation included not only butyl alcohol, but also isopropyl alcohol and small amounts of *n*-propyl and isobutyl alcohols, and acetic, butyric, and isobutyric acids.

Morikawa[2] isolated an organism designated as *Bacillus technicus* Prescott and Morikawa, which produced from *koji* mashes butanol; isopropyl alcohol; traces or small amounts of acetone, acetic, and butyric acids; and hydrogen and carbon dioxide. This organism appeared to differ in several respects from the organism described as *Cl. americanum* Pringsheim. The fermentation brought about by *B technicus* has been studied by Morikawa and Prescott,[3] and by Dunn.[4]

Cl butylicum (Beijerinck) Donker was studied by Van der Lek.[5]

Considerable research concerning various aspects of the butyl-isopropyl alcohol fermentation has been carried out by Osburn;[6] Osburn and Werkman;[7] Osburn, Brown, and Werkman;[8] Langlykke, Peterson, and McCoy;[9] Langlykke, Peterson, and Fred,[10] Sjolander, Langlykke, and Peterson;[11] and others.

Some General Considerations concerning the Fermentation.—There are several organisms that have the ability to produce isopropyl alcohol. These are closely related In view of the fact that *Cl. butylicum* (Beijerinck) Donker is perhaps the best known of the isopropyl alcohol producers, this organism and the fermentations it produces will be considered in some detail.

Description of Cl. butylicum [12]—*Cl butylicum* is a sporeforming obligate anaerobe, possessing peritrichous flagella It is Gram-positive in young cultures but in old cultures may be Gram-negative The vegetative

[1] FOLPMERS, T , *Tijdschr Vergelijk Geneeskunde*, Nov 5–7 (1920–1922)

[2] MORIKAWA, K , "A New Butyl and Isopropyl Alcohol Fermentation," Thesis, Massachusetts Institute of Technology, Cambridge, 1926

[3] MORIKAWA, K , and S C PRESCOTT, *Jour. Bact*, 13: 58 (1927)

[4] DUNN, C G , "A Biochemical Investigation of the Metabolic Activities of *Bacillus technicus*," Thesis, Massachusetts Institute of Technology, Cambridge, 1934

[5] VAN DER LEK, J B , Thesis, Delft, 1930

[6] OSBURN, O L , *Iowa State Coll Jour Sci*, 10: 97 (1935)

[7] OSBURN, O L , and C H WERKMAN, *Ind Eng Chem*, 27: 416 (1935)

[8] OSBURN, O L , R W BROWN, and C H WERKMAN, *Jour Biol Chem*, 121: 685 (1937); *Iowa State Coll Jour Sci*, 12: 275 (1938)

[9] LANGLYKKE, A F , W H PETERSON, and C McCOY, *Jour Bact*, 29: 333 (1935)

[10] LANGLYKKE, A F , W H PETERSON, and E B FRED, *Jour Bact*, 34: 443 (1937).

[11] SJOLANDER, N O , A F LANGLYKKE, and W H PETERSON, *Ind Eng Chem*, 30: 1251 (1938)

[12] Courtesy of Osburn, Brown, and Werkman, *Iowa State Coll Jour Sci*, 12: 275 (1938)

cells of a 24-hr. culture grown at 37°C. in a 4 per cent corn mash containing potato extract are rod-shaped, 2 to 5 μ in length and 0.7 to 1.5 μ in width. These cells, which have rounded ends, occur singly, in pairs, or in chains. Granulose is present in young cells. Old cultures contain many spores.

The optimum temperature for growth is 37°C.

Cl. butylicum does not produce indol; reduce nitrates to nitrites; liquefy starch; assimilate peptone, unless carbohydrates are present; or utilize ammonium salts. It is catalase negative and forms hydrogen sulphide from sulphites, thiosulphites, and oatmeal.

Acid and gas are formed from amygdalin, arabinose, cellobiose, dextrin, dimethylglucoside, esculin, galactose, glucose, glycogen, inositol, inulin, lactose, levulose, maltose, melibiose, melezitose, raffinose, rhamnose, salicin, starch, sucrose, trehalose, and xylose. No acids or gas are formed from adonitol, dulcitol, erythritol, glycerol, mannitol, pectin, sodium lactate, or sorbitol.

The final products include butyl and isopropyl alcohols, carbon dioxide and hydrogen, small quantities of butyric and acetic acids, and possibly traces of acetone and formic acid.

Granulobacter butylicum (Beijerinck) and *Cl. americanum* (Pringsheim) are names now considered to be synonyms of *Cl. butylicum* (Beijerinck) Donker.

Nitrogen Sources.—Some of the most favorable sources of nitrogen for the butyl-isopropyl alcohol organisms are yeast extract, peptone, malt sprouts, and corn steep. Each of these nitrogenous substrates contains protein in a partially hydrolyzed form. Other partially hydrolyzed proteins may be used as sources of nitrogen. *Cl. butylicum* does not hydrolyze proteins appreciably.

Malt sprouts have been used by Beijerinck, Folpmers, Osburn and Werkman, and others. One sample of malt sprouts used by Osburn and Werkman[1] contained 3.52 per cent of nitrogen, 10 per cent of maltose, and 18 per cent of starch and dextrin. Of the nitrogenous material, 35 per cent was soluble in hot water, and 30 per cent was in the form of amino nitrogen.

During the processing of corn in the manufacture of corn products, the clean corn is steeped for approximately 2 days in warm water containing a small quantity of sulphur dioxide. Soluble materials are extracted. The steep water is concentrated to approximately 12°Bé and is known as "corn steep." In some of their work, Osburn and Werkman[1] used a sample of corn steep water containing 2.896 per cent of nitrogen. When the water was neutralized, 34 per cent of the nitrogenous substance

¹ Osburn and Werkman, *loc. cit.*

precipitated out. Of the total nitrogen left, 32 per cent was in the form of amino and amide nitrogen, and it was assumed that the remaining 34 per cent was contained in partially hydrolyzed protein and albumins.

Osburn and Werkman[1] demonstrated that from 5 to 11 per cent of isopropyl alcohol and from 19.5 to 25 per cent of butanol could be produced from 4 per cent glucose media containing mixtures of corn steep water and malt sprouts or corn gluten (the proteins of corn gluten are zein and glutelin principally)

Low yields of butanol (3 to 4 per cent) were obtained in the fermentation of 4 per cent corn mashes by *Cl butylicum*, but the addition of 1 g. of yeast extract, or 5 cc. of corn steep water, per 300 cc. of mash resulted in vigorous fermentations that went to completion with the production of 12 to 14 per cent of butanol [2]

The addition of asparagine,[3] yeast extract, or peptone to 5 per cent corn mashes caused large yields of butanol to be formed in place of butyric acid by *Cl. butylicum* The stimulating effect of asparagine was proportional to the amounts used up to a concentration of 0.4 g. of asparagine per 300 cc. of 5 per cent corn mash

Composition of Some Media.—In some of their research, Osburn and his associates[4] used a basal medium of the following composition:

	Per Cent
Glucose	2
Peptone	0 7
Difco yeast extract (powdered)	0 2
Dipotassium hydrogen phosphate	0 1

Langlykke and his coworkers[5] employed a medium containing the following ingredients

	Per Cent
Glucose	3 (approx)
Peptone	0 5
Asparagine	0 1
Dibasic ammonium phosphate	0 07

Neither peptone nor asparagine was satisfactory as the sole source of nitrogen.

[1] OSBURN and WERKMAN, *loc cit.*

[2] OSBURN and WERKMAN, *op cit*, 27: 416–419 (1935)

[3] BROWN, R W, G L STAHLY, and C H WERKMAN, *Iowa State Coll Jour*, *Sci*, 12: 215–251 (1938)

[4] OSBURN, O L, R W BROWN, and C H WERKMAN, *Jour. Biol Chem*, 121: 685–695 (1937)

[5] LANGLYKKE, PETERSON, and FRED, *loc cit*

Isopropyl Alcohol from Wood Sugars.—The utilization of wood sugars in the production of butyl and isopropyl alcohols has been studied by Sjolander, Langlykke, and Peterson.[1]

The wood hydrolyzates, similar to some obtained in the Scholler process (Chap. IV), were treated to remove inhibitory substances. Excess calcium carbonate was added to neutralize the acidity of the hydrolyzates. The precipitate of calcium sulphate formed was removed by filtration, and the filtrate was adjusted to a pH of approximately 10 with lime to cause precipitation of iron and copper. Again the medium was filtered. The filtrate was acidified to a pH of about 6.5, clarified with norite, and adjusted to a pH of 6.0.[2]

As a result of experimentation, a nutrient medium containing the following constituents was used by Sjolander, Langlykke, and Peterson:

		Per Cent
Malt sprouts	. . .	2
Dried whole liver..	. . .	0.25
Dibasic ammonium phosphate	. . .	0 05
Treated wood sugar solution	.	As indicated
Calcium carbonate	.	0.1

Calcium carbonate was sterilized separately and added, using aseptic precautions, to the sterilized medium. The inoculated mashes were incubated at 37°C. for 5 days under anaerobic conditions.

TABLE 78.—PRODUCTS OF FERMENTATION BY *Cl. butylicum*[1]

Carbohydrate source	Sugar as glucose		Neutral volatile products, per cent[2]	Distribution of neutral volatile products				Volatile acids, milligrams per cubic centimeter[3]	
	Original concentration, per cent	Fermented, per cent		Butyl alcohol, per cent	Ethyl alcohol, per cent	Acetone, per cent	Isopropyl alcohol, per cent	Acetic	Butyric
Hemlock 10	3 15	92	34 6	59	9	4	28	2 4	1 3
Beech 11	2 83	90	33.2	53	8	4	35	4 8	3 3
Glucose	2 90	99	29.8	70	7	3	20	1 0	1 1
Beech 11 (ether-extracted)	2 99	75	26 8	68	10	3	19	1 1	1 8
Glucose + acetate	2 95	98	28 7	49	1	9	41	4 3	2 7

[1] Sjolander, N. O., A. F. Langlykke, and W. H. Peterson, *Ind. Eng. Chem.*, 30: 1251–1255 (1938).
[2] Based on the apparent sugar fermented
[3] Volatile acids in uninoculated media (in milligrams per cubic centimeter)

[1] Sjolander, Langlykke, and Peterson, *loc. cit.*
[2] Ibid

	Acetic	Butyne
Hemlock 10	4 4	0
Beech 11	9 0	0 2
Beech 11 (ether-extracted) . . .	0 4	0
Glucose + acetate	8 1	0 1

The results of some experiments, using *Cl. butylicum*, are given in
Table 78.

An examination of the foregoing table indicates that more isopropyl
alcohol was formed from hemlock 10 and beech 11 wood sugars than from
glucose. It was assumed that the higher yields of isopropyl alcohol from
the media containing the wood hydrolyzates were due to the acetic acid
contents of the uninoculated media. These contents were reduced
during the fermentation

In order to ascertain the correctness of the assumption that the
increased yields of isopropyl alcohol were due to the acetic acid present
in the uninoculated media, Sjolander and his associates carried out two
experiments. In one experiment beech 11 wood hydrolyzate was
extracted for 48 hr continuously with ether This ether-extracted
medium was thus nearly freed of volatile acids Table 78 shows that
the products formed by fermentation of this medium were similar quanti-
tatively to those produced from glucose In the other experiment, a
quantity of calcium acetate, equivalent to the acetic acid of the unex-
tracted beech 11 wood hydrolyzate, was added to a medium containing
glucose as the source of sugar The percentage of isopropyl alcohol
produced was over twice that produced in the glucose solution con-
taining no calcium acetate, but the yield of butanol was considerably
smaller.

Yields of End Products.—The quantities of end products formed from
glucose by *Cl. butylicum* in a series of laboratory experiments are shown
in Table 79

Effect of Neutralization with Sodium Bicarbonate.—Osburn and his
associates[1] added sodium bicarbonate to fermenting glucose mashes with
the result that butyl alcohol and isopropyl alcohol production was almost
completely suppressed, the formation of isopropyl alcohol being inhibited
more markedly than that of butanol In the presence of relatively large
concentrations of sodium bicarbonate, salts of acetic, butyric, formic,
lactic, and pyruvic acids accumulated in the mash

In carrying out the experiments, Osburn, Brown, and Werkman

[1] Osburn, O I , R W Brown, and C H Werkman, *Jour Biol Chem*, 121: 685 (1937)

TABLE 79.—FERMENTATION OF GLUCOSE BY *Cl butylicum*[1]
(Medium: 2 per cent cane solution with 0 7 per cent peptone, 0 2 per cent yeast extract, and 0 1 per cent dipotassium phosphate)[1]

Experiment no.	Products per 100 millimols fermented								
	Alcohols			Acids		Carbon dioxide, millimols	Hydrogen, millimols	Carbon recovered, per cent	Oxidation-reduction index[2]
	Butyl, millimols	Iso-propyl, millimols	Ethyl, millimols	Butyric, millimols	Acetic, millimols				
1	57 6	15.1	2.9	15.1	9.1	166.5	75.8	87 0	0 86
2	66 6	15.1	0	6.1	21.2	197.0	72.7	95.0	1.02
3	58 6	12 1	0	17.2	17.2	203.5	77.6	96.3	1 06
4	54 6	15 1	2.9	18.2	12.1	200.0	121.2	93.5	0 93
5	65 5	13 8	2.3	13.8	10.3	189.5	86.2	96 5	0 90
6	50 2	18 0	0	14.5	20.3	207.0	111.1	93.5	1.05
7	54 1	18 0	0	15.5	12.9	222.0	107.0	96 2	1.08

[1] Osburn, O. L., R. W. Brown, and C. H. Werkman, *Jour. Biol. Chem.*, 121: 685 (1937).
[2] Ratio of oxidized to reduced products; a perfect ratio = 1.0 (cf Erb, Wood, and Werkman, *Jour Bact*, 31: 595 (1936), for method of calculation).

added an 8 per cent solution of sodium bicarbonate to the inoculated glucose mashes after the fermentations had become vigorous, usually after about 14 hr. In each case, the solution was added rapidly at first and then gradually until the weight of sodium bicarbonate added became

TABLE 80.—FERMENTATION OF 2 0 PER CENT GLUCOSE BY *Cl. butyleum*[1]
(In presence of sodium bicarbonate, 0 7 per cent peptone, 0 2 per cent yeast extract, 0 1 per cent dipotassium phosphate)[1]

Experiment no.	Products calculated per 100 millimols glucose fermented											
	Alcohols		Acids					Carbon dioxide, millimols	Hydrogen, millimols	Carbon recovered per cent	Oxidation-reduction index	pH
	Butyl, millimols	Iso-propyl, millimols	Butyric, millimols	Acetic, millimols	Pyruvic, millimols	Lactic, millimols	Formic, millimols					
1	3 0	0	54 0	24 0	3 0	43 5	0	135 0	135 9	91 7	1 05	7 2
2	3 0	3 0	51 0	33 0	12.0	37 8	0	135 0	150 0	89 4	1 21	6 7
3	5 4	1 8	33 7	27 0	11 2	36 2	27 0	91 5	87 4	92 0	1 35	6 9
4	6 7	1 9	30 6	35 4	7 6	60 2	47 3	74 6	87.9			

[1] Osburn, O. L. R. W. Brown, and C. H. Werkman, *Jour. Biol. Chem.*, 121: 685 (1937).

equal to the weight of the sugar being fermented. The results of these experiments are shown in Table 80.

Methylglyoxal was isolated from the fermenting mashes and identified.

Isopropyl Alcohol Production by Bacillus technicus.—An organism isolated from *koji* rice and described in 1926 by Morikawa[1] produced butyl and isopropyl alcohols, acetic and butyric acids, carbon dioxide

TABLE 81.—FERMENTATIONS CHARACTERIZED BY THE PRODUCTION OF ISOPROPYL ALCOHOL[1]

Culture no.	Glucose fermented,[2] per cent	Acidity 0 1 N NaOH in 10 cc., cc.	Neutral volatile products based on glucose fermented				
			Butyl alcohol, per cent	Ethyl alcohol, per cent	Iso-propyl alcohol, per cent	Acetone, per cent	Total, per cent
21	94 9	3.95	19 2	2 5	4 7	0 5	26 9
22	94.9	3.70	22 2	2 2	4 4	0.7	29 5
36	94 1	3 75	19 3	2.3	5 2	0 0	27 7
18	78.0	5.30	17.8	2 1	4.5	0.6	25.0
24	75.1	4 85	16.6	2.8	6 1	0 8	26 3
20	94.8	4 30	15.7	2.4	3 2	1 6	22 9
30	93.1	3 65	21 0	1.7	3 8	1 3	27 8
46	94.5	3 30	17 1	2 4	3 8	3.7	27 0

[1] LANGLYKKE, A. F., W. H. PETERSON, and E. McCOY, *Jour. Bact.*, **29**: 333–347 (1935)
[2] The medium consisted of a double-strength yeast water ("the clear water extract of fresh, starch-free yeast, 200 g per liter of tap water") containing 2 5 per cent glucose with the reaction adjusted to pH 7 0.

and hydrogen and traces or small amounts of acetone as the principal end products from suitable nutrient carbohydrate media Hydrolyzed *koji* rice; malt sirup; cerelose; mixtures of dextrins, maltose and glucose; or mixtures of hydrolyzed *koji* rice with certain other sugars were readily fermented. Unhydrolyzed corn mashes, purified glucose or maltose, and blackstrap molasses were poorly fermented with low yields. The optimum concentration of sugar was 10 to 13 per cent, the optimum reaction, pH 5 to 7. Good yields were obtained at temperatures of 28 to 37°C. Calcium carbonate stimulated the formation of neutral solvents.

Isopropyl Alcohol Production by Butyric Acid Anaerobes.—Some butyric acid anaerobes produce isopropyl alcohol However, the principal neutral solvent formed is butyl alcohol. Ethyl alcohol is formed in

[1] MORIKAWA, *loc. cit*; PRESCOTT, S. C., and K MORIKAWA, U.S Patent 1,933,613, Nov. 7, 1933

CHAPTER XV

THE ACETIC ACID BACTERIA AND SOME OF THEIR BIOCHEMICAL ACTIVITIES

The acetic acid bacteria belong to the family *Pseudomonadaceae*.[1] The cells are rod-shaped, but elongated, filamentous, club-shaped, swollen, or branched forms may occur. They may be motile or nonmotile and do not form endospores. The bacteria may secure energy by the oxidation of ethanol to acetic acid, by the oxidation of various sugars and other alcohols, or by anaerobic dissimilations.

The following members of the genus *Acetobacter* are listed and described in "Bergey's Manual":[1]

> *Acetobacter aceti* (Kützing) Beijerinck (the type species)
> *A. pasteurianum* (Hansesn) Beijerinck
> *A. kuetzingianum* (Hansen) Bergey et al
> *A. zeidleri* Beijerinck
> *A. acetosum* (Henneberg) Bergey et al.
> *A. xylinum* (Brown) Bergey et al.
> *A ascendens* (Henneberg) Bergey et al.
> *A. plicatum* Fuhrmann
> *A. acetigenum* (Henneberg) Bergey et al.
> *A. oxydans* (Henneberg) Bergey et al.
> *A industrium* (Henneberg) Bergey et al.
> *A. rancens* Beijerinck
> *A. melanogenum* Beijerinck
> *A. suboxydans* Kluyver and de Leeuw
> *A viscosum* Shimwell
> *A capsulatum* Shimwell
> *A. gluconicum* (Hermann)
> *A turbidans* Cosbie, Tošić, and Walker
> *A peroxidans* Visser't Hooft

Henneberg has described the five following species of acetic acid bacteria, which are listed in "Bergey's Manual":

> *Bacterium schuetzenbachii* Henneberg
> *Bact xylinoides* Henneberg
> *Bact orleanense* Henneberg
> *Bact. vini acetati* Henneberg
> *Bact. curvum* Henneberg
> *Bact. dihydroxyacetonicum* Virtanen and Barlund

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[1] "Bergey's Manual of Determinative Bacteriology," 6th ed., The Williams & Wilkins Company, Baltimore, 1948.

Biochemical Activities of the Acetobacter.—The biochemical activities of the *Acetobacter* consist mainly of aerobic and anaerobic dissimilations and the synthesis of polysaccharides. The aerobic dissimilations are, from the industrial viewpoint, most important, including the oxidative dissimilation of sugars and alcohols. The oldest and best known of the fermentations brought about by acetic acid bacteria is that in which acetic acid or vinegar is produced.

VINEGAR

Vinegar may be defined as the condiment made from sugary or starchy materials by alcoholic and subsequent acetous fermentations. The term literally signifies "sour wine," according to its derivation from the French (*vinaigre* = *vin*, "wine," plus *aigre*, "sour" or "sharp").

Composition.—The composition of a vinegar will depend somewhat on the nature of the raw material that has undergone alcoholic and acetous fermentations. The conditions of manufacture, aging, and storage will also influence the composition of the product. In a cider vinegar, for example, one might find, besides at least 4 g. of acetic acid (CH$_3$-COOH) per 100 cc. of vinegar at 20°C., traces or small amounts of alcohol, glycerin, esters, reducing sugars (as invert sugar), pentosans, salts, and other substances.[1]

The Food and Drug Administration of the United States[2] has adopted the following definitions and standards for vinegars.

Vinegar, cider vinegar, apple vinegar. The product made by the alcoholic and subsequent acetous fermentations of the juice of apples. It contains, in 100 cubic centimeters (20°C.), not less than 4 grams of acetic acid.

Wine vinegar, grape vinegar. The product made by the alcoholic and subsequent acetous fermentations of the juice of grapes. It contains, in 100 cubic centimeters (20°C), not less than 4 grams of acetic acid.

Malt vinegar. The product made by the alcoholic and subsequent acetous fermentations, without distillation, of an infusion of barley malt or cereals whose starch has been converted by malt. It contains, in 100 cubic centimeters (20°C.), not less than 4 grams of acetic acid.

Sugar vinegar. The product made by the alcoholic and subsequent acetous fermentations of sugar sirup, molasses, or refiners sirup. It contains, in 100 cubic centimeters (20°C.), not less than 4 grams of acetic acid.

Glucose vinegar. The product made by the alcoholic and subsequent acetous fermentations of a solution of glucose, is dextrorotatory and contains, in 100 cubic centimeters (20°C.), not less than 4 grams of acetic acid.

[1] Brooks, R. O., "Critical Studies in the Legal Chemistry of Foods," Reinhold Publishing Corporation, 1927

[2] U.S. Dept. of Agriculture, F.D.A., Service and Regulatory Announcements, Food and Drug, No. 2, Rev. 5, November, 1936

Spirit vinegar, distilled vinegar, grain vinegar. The product made by the acetous fermentation of dilute distilled alcohol. It contains, in 100 cubic centimeters (20°C.), not less than 4 grams of acetic acid.

Historical.—Although vinegar has been known for thousands of years, its microbiological nature was not realized until a little more than 100 years ago, when Kützing (1837) reported that the conversion of ethanol to acetic acid was brought about by living microorganisms Fifteen years earlier, Persoon had given the name *Mycoderma* to the film that formed on liquids in which an acetic fermentation was taking place

It remained for Pasteur (1868) to confirm Kützing's opinion and to prove the physiological nature of the acetic acid fermentation. Pasteur, however, believed that the fermentation was caused by a single species of bacteria, *Mycoderma aceti.* In 1878, Hansen showed that more than one species of bacteria could bring about the souring of beer, *i.e.,* the oxidation of the ethanol to acetic acid. He isolated and named *Bacterium aceti* and *Bact. pasteurianum.* At a later date he isolated *Bact kutzingianum,* while a fourth species was described by Brown. About 1897 Henneberg studied and reclassified the group and described several other species.

Nomenclature.—The literature contains occasionally more than one name for the same species of *Acetobacter.* For example, *A. aceti* has been referred to both as *M aceti* and *Bact. aceti.* The system of nomenclature adopted in "Bergey's Manual" will be used in this chapter.

General Requirements for Manufacture.—In the manufacture of vinegar several factors are worthy of special consideration: the selection of the microorganism; the nature of the raw material; the concentration of the ethanol used, as well as that of the vinegar added at the start to acidify it; the amount of oxygen supplied; the nature of the supporting medium; the temperature of the fermentation; aging and storage, clarification; bottling and pasteurization; and the character and composition of the tanks, containers, and fixtures coming in contact with the vinegar during the manufacturing process.

Selection of Microorganism.—Although there are a large number of bacteria, as well as other microorganisms, that have the ability to produce acetic acid in small amounts from various substrates, only relatively few bacteria possess the characteristics desired for vinegar production *Bact. schuetzenbachii* or *Bact. curvum* may be used to produce acetic acid from ethyl alcohol in the quick vinegar process, while *Bact. orleanense* may be used in either the quick vinegar or Orleans process. *Acetobacter aceti, A. pasteurianum, A. xylinum, A. ascendens,* and *A. acetigenum* may be isolated from vinegar

Nature of Raw Material.—Vinegar may be manufactured from almost any product capable of yielding alcohol by fermentation. Fruits such as apples, grapes, pears, peaches, plums, figs, and oranges; berries; honey; sugar-containing sirups; hydrolyzed starchy materials; beer; and wine may serve as raw materials for vinegar manufacture.

Wine and apple juice, or cider, are two of the best raw materials for vinegar production—wine being used to a large extent in France, Italy, Spain, and Greece; cider in the United States. Vinegar prepared from malt is popular, especially in England, while that from honey is considered to be very palatable.

It is obvious that the quality of the vinegar will depend in large part on the quality of the raw material used. Fruit should be clean, sound, and in the proper state of maturity; wine or alcoholic media should be clear, clean, and free from preservatives. Cleanliness of the plant, equipment, and surroundings are likewise indispensable for the production of a high-grade product.

Yeast Fermentation.—Before the acetic acid fermentation can take place, the sugar in the fruit juice or other sugar-containing medium must be converted to alcohol by yeast fermentation. The yeast naturally present in the fruit juice may bring about a successful spontaneous fermentation, but the manufacturer cannot rely on chance and should use a starter in order to ensure a suitable fermentation. Although compressed yeast may be often used satisfactorily as a starter, the use of a selected wine yeast, for example, *Saccharomyces ellipsoideus*, generally improves the flavor of the final product. Fermentations may be carried out favorably at 75 to 80°F. (23.9 to 26.7°C.)[1] It is advisable to follow the course of the fermentation with hydrometers (Brix or Balling) that indicate the approximate percentage of sugar present in the fermenting mash. When the fermentation is complete, yeast, pulp, and other sediment should be removed from the medium by a process of settling. A storage period of 2 to 3 weeks is usually allowed for the sedimentation, after which the clear medium is "racked" (drawn off), adjusted, if necessary, to the optimum alcohol concentration, and acidified by the addition of some pure vinegar.

Concentration of Alcohol.—Adjustment of the alcohol content of the medium may be necessary in order to ensure a successful fermentation. Alcohol in a concentration of 10 to 13 per cent is readily fermented. When using alcohol concentrations of 14 per cent or greater, the zoogloeal mat forms with difficulty and the alcohol is incompletely oxidized to acetic acid. On the other hand, the use of too low concentrations may result in the loss of vinegar, for, when the concentration of ethanol is less

[1] CRUESS, W. V., and M. A. JOSLYN, *Calif. Agr. Expt. Sta., Circ.* 332, 1934.

than 1 or 2 per cent, esters and acetic acid are oxidized with the loss of aroma and flavor. Carbon dioxide and water are formed from acetic acid:

$$CH_3COOH + 2O_2 \rightarrow 2CO_2 + 2H_2O$$
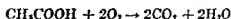
With some species of acetic bacteria this action may occur even in normal concentrations.

Acidification.—The initial acidification is carried out with two objects in view: to inhibit the development of undesirable types of bacteria and to supply desirable acetic-acid-producing bacteria for seed purposes. The amount of vinegar added to the alcoholic medium will depend upon the nature of the process, but usually 10 to 25 per cent by volume of strong vinegar is considered to be sufficient. If the mix is to be permitted only one passage through a generator, the initial acidity may be adjusted to 3 to 3.5 per cent and sufficient alcoholic substrate added to yield a vinegar with a final acid strength of approximately 6 per cent.[1]

A medium should never be acidified before the alcoholic fermentation is complete, because the sugar in the medium would be incompletely converted to alcohol after the addition of acetic acid. Vinegars made from incompletely fermented juices are usually low in acetic acid and of poor quality.

Oxygen.—Since the conversion of ethanol to acetic acid is primarily an oxidation process, or a dehydrogenation in which atmospheric oxygen acts as the hydrogen acceptor, the success of the fermentation will depend in no small part on the availability of large quantities of oxygen. The following equations will illustrate the requirement for oxygen:

$$CH_3CH_2OH + \tfrac{1}{2}O_2 \rightarrow CH_3CHO + H_2O$$
$$\text{Ethanol} \qquad\qquad \text{Acetaldehyde}$$
$$CH_3CHO + \tfrac{1}{2}O_2 \rightarrow CH_3COOH$$
$$\text{Acetaldehyde} \qquad\qquad \text{Acetic acid}$$
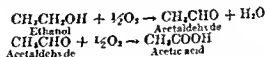
If fermentation is to go on in casks or barrels they should be provided with an adequate number of holes above the surface of the liquid to ensure a plentiful oxygen supply. The openings should be screened to keep out insects, and the barrels should be only partly filled. When generators are used they should be packed rather loosely with zoogloea-supporting materials to permit sufficient ventilation in all parts of the generator.

Commercial production of vinegar and acetic acid by fermentation is usually carried out in large casks of proper design or in generators constructed in the form of a truncated cone provided at top and bottom with

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[1] CRUESS, W. V., "Commercial Fruit and Vegetable Products," 3d ed., McGraw-Hill Book Company, Inc., New York, 1948.

perforated scaffolds, and near the bottom with air inlets. The generator thus acts as a stack or chimney in which a strong upward current of air takes place, bringing the oxygen in contact with the bacterial film resident on the surfaces of the supporting material for the film (In the section on methods of manufacture, the construction will be taken up in greater detail.)

The Supporting Medium.—The use of a satisfactory supporting medium has much to do with the duration and success of the acetic fermentation, since its purpose is to increase greatly the area or surface exposed and thus accelerate the fermentation by virtue of the availability of a larger oxygen supply

Many manufacturers of vinegar during and before the time of Pasteur failed to realize the necessity for an adequate oxygen supply and the use of a support for the film in containers where the zoogloeal mat was likely to be disturbed periodically Disturbed films sank and used up the nutrient materials anaerobically without producing acetic acid. Pasteur pointed out defects in the processes and made suggestions as to how they could be improved.

A "raft" or light wooden grating may be used as a support for the bacterial film in the cask or barrel In the rapid fermentation process, where generators are used, the supporting medium is usually constructed of beechwood shavings or chips, although rattan, wood charcoal, coke, pressed pomace, corncobs, excelsior, or other materials that offer large surface areas may be substituted Coke is more durable than wood charcoal, while corncobs are not particularly durable

It is essential that the material used for supporting purposes should impart no undesirable odors or flavors to the vinegar The material should be thoroughly extracted with water and then with vinegar before it is used in the generator.

Temperature Relations of the Acetic Acid Bacteria.—The acetic group of bacteria is characterized by very definite and peculiar temperature relations. At temperatures below 12 to 15°C it grows slowly, and the cells are short but unusually broad From 15 to 34°C they appear to develop in what may be called the "normal" manner, growing rapidly and developing chains of cells of varying number of units or elements. In suitable media the walls become swollen and exhibit the early stages of zoogloea formation. At still higher temperatures (approximately 42 to 45°C) long thread-like transparent filaments with no cross walls and with irregular bulging and occasional branching have been observed This condition appears to be a pathological state induced by high temperature, and if the culture is long maintained under these conditions it may lose its power to function normally A prompt return to tempera-

bunghole likewise screened. The acetic acid bacteria form a thin film on the surface of the solution, and this film later becomes quite thick and gelatinous. This gelatinous zoogloeal mat, which contains very large numbers of bacteria, is known as the "mother of vinegar." Eventually, unless supported on a "raft" or framework, it will sink to the bottom of the barrel and a new film will form.

Although vinegar of high grade is produced by this method, it is a slow and costly process that involves much attention. The films are easily disturbed by the addition of the alcoholic medium and the withdrawal of vinegar. If they sink, they use up the nutrient substances but fail to produce acetic acid under anaerobic conditions. Pasteur made suggestions as to how to improve this process. One such suggestion involved the use of a support for the film.

Modifications of the Orleans Process.—Most of the slow methods are modifications of the old Orleans process. A light grating of wood may be floated on the liquid medium to support the bacterial film and prevent it from breaking up and sinking. Another method[1] to prevent submergence of the film is to equip the top bunghole with a funnel attached to a glass tubing that leads to the bottom of the barrel. Alcoholic solutions can be added with a minimum disturbing effect on the film. A glass tube may be attached through a bunghole in the bottom of the barrel to serve as a gauge for measuring the level of the liquid in the barrel during fermentation and as a means for withdrawing finished vinegar without disturbing the zoogloeal mat.

The Quick Vinegar Process.—The quick vinegar process, now largely used, is also known as the "German process." Boerhave discovered in the early part of the nineteenth century that when wine was permitted to trickle down through a tall receptacle containing loosely packed pomace, vinegar was rapidly produced. Schutzenbach (1823) modified the method of Boerhave by introducing other types of porous material in order to obtain maximum contact of the organisms with air. The method used by Schutzenbach is the basis for modern methods of manufacture using the generator.

Generators.—Generators are of various sizes and shapes. Some are 10 ft. in diameter and 20 ft. high. Some are 8 by 16 ft. or 4 by 8 ft.[2] The generator is equipped with a false perforated bottom, through which air enters. Some of the larger generators have a perforated shelf approximately halfway between the top and the bottom of the tank, which aids in supporting the beechwood shavings or other material used to present a

[1] *Ibid.*

[2] FETZER, W. R., *Food Industries*, **2**: 489 (1930).

large surface area for the acetic acid bacteria. The use of the perforated shelf prevents crushing and matting of the shavings due to the weight of the superimposed material. Near the top of the generator above the shavings there is a false top or perforated plate over which is arranged a rotating sprinkler, or sparger, for producing a uniform distribution of the vinegar stock (vinegar plus alcohol-containing substrate) over the top
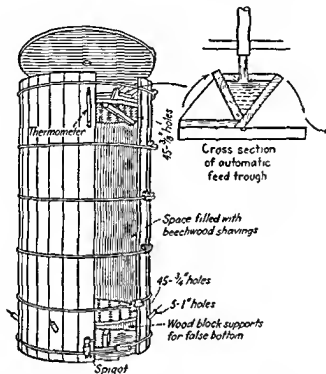
Fig. 63 —Generator for quick process. *(Courtesy of E LeFevre, U.S. Dept Agr , Farmers' Bull 1424, 1936)*

surface of the supporting material. In place of a sparger at the very top of the generator there may be located a tilting trough or other automatic device, which periodically dumps vinegar stock upon the distributing head

The vinegar stock may be passed through the same generator until the desired acidity is obtained, or it may be passed through two or three sets of generators connected in series, each with increasing acidity The latter method of operation is known as "tandem operation "

A generator 10 ft in diameter and 20 ft high usually produces 80 to 100 gal. of distilled vinegar per day [1]

[1] Ibid

The Frings Method.—Vinegar may be manufactured by the Frings process.[1] This process, in fundamentals, is similar to the quick generator process, but it possesses several advantages, which will be mentioned later
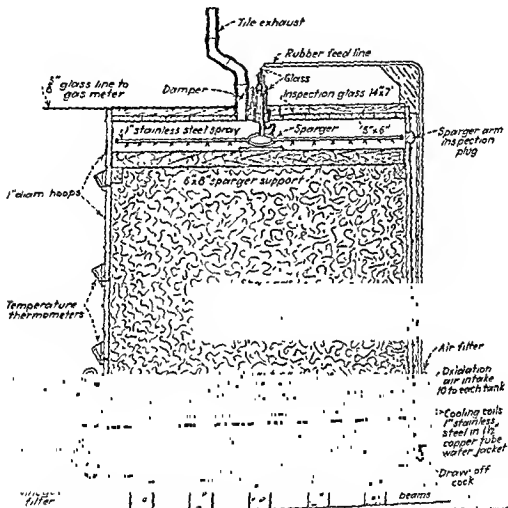
FIG 64—Cross-section of the Frings generator   [Courtesy of A. E. Hansen, Food Industries, 7:277 (1935)]

FRINGS GENERATOR —Figure 64 shows a cross-section of the Frings generator   The generator consists of an airtight tank, which is usually 14 ft. in diameter and 15 ft. in height, and certain accessory equipment. The tank is superimposed on concrete beams, in such a manner that air may circulate beneath the apparatus.   Inside the tank, near the bottom, is a wooden grating, which supports beechwood shavings.   These shav-

[1] HANSEN, A. E., *Food Industries*, 7:277 (1935), FRINGS, H , U S Patent 1,880,381, Oct 4, 1932

ings are piled to within about 1.5 ft. of the top of the generator. Below the wooden grating at the bottom of the tank is the collection chamber of the generator. At the very bottom of this chamber is a cock or faucet through which the finished product is withdrawn. Near the bottom of the tank, cooling coils are also located. The vinegar mix, which has passed down through the shavings, is circulated through an inner pipe made of stainless steel, or other acid-resistant substance, and is cooled by the passage of water through a surrounding outer pipe of copper. The mix is then forced by means of a centrifugal pump made of stainless steel from the cooling coils to the top of the tank through a rubber line. The mix passes from the rubber feed line through a glass connecting device to a sparger, or distributing arm, constructed of stainless steel

The oxygen supply of the generator is controlled by a damper, located in a 4-in vent constructed at the top of the tank. This vent constitutes the air outlet of the generator. Air enters the generator through a series of intakes (10) located around the tank near the level of the wooden grate. These inlets contain air filters

Thermometers are located at different points in the generator in order to determine the temperatures in the different parts.

The generator contains a meter, attached near the pump, for measuring the rate of flow of the mix or medium, also a meter for recording the rate of flow of the cooling water

A generator of the type just described may produce approximately thirty times the quantity of white vinegar that can be produced by a tank (4 by 8 ft) of the noncirculating type.[1]

COMPOSITION OF THE MIX.—A 2,500-gal. mix may contain 10 5 per cent of ethanol, 1 per cent of acetic acid, and 7 lb. of a special medium for acetic acid bacteria, known as "aceto-pep" (devised by Frings).

OPERATION OF THE GENERATOR —The mix is permitted to circulate repeatedly through the beechwood shavings until vinegar of the desired strength has been obtained During the process heat is generated, and large quantities of oxygen are consumed. Since the tank is airtight, except for the air inlets and vent, the continued evolution of heat would produce a temperature sufficiently high to inhibit the action of the acetic acid bacteria. By passing the nutrient acid-alcohol mix through the cooling coils, the temperature of the mix is carefully controlled. The cooled mix is returned to the sparger and sprayed over the surface of the shavings. It trickles down through the shavings, acetic acid being formed from the ethanol. It is again cooled and recirculated. This cycle is repeated until the acetic acid content of the mix has become 105

grains.   It may require 8 to 10 days for the conversion of the ethanol in a 2,500-gal. batch to acetic acid of this concentration.   The vinegar is then withdrawn, except for about 200 gal., which is left as food for the bacteria and as a primer for the pump.   A new batch of mix is promptly run into the generator.

ADVANTAGES OF THE PROCESS.—This process has several advantages. It may be operated at a low cost and it is easily controlled and relatively simple.   Vinegars of higher acetic acid concentrations than those produced by other types of generators may be produced.   The tank utilizes less space than that required by other types of generators for the production of the same quantity of vinegar in the same time.   Losses due to evaporation and to the presence of fumes in the room are avoided, for the generator is airtight.   The temperature of the room does not affect the temperature of the interior of the generator adversely.   Owing to continuous operation, there is little tendency for slime to form in the generator.

Vinegar Generator with Automatic Control.—A vinegar generator system has been designed by Elmer[1] in which automatic control is exercised and in which the efficiency of yield is high.   The vinegar mix flows by gravity from an elevated reservoir through a fluid course to the generator and thence to a storage reservoir.   The fluid course is provided with a vessel for measuring the mix.   A vertically adjustable float in the measuring vessel actuates a cock to cause the mix to flow in from the elevated reservoir.   The mix is automatically discharged from the measuring vessel to the generator through the agency of a valve electrically actuated by an electric circuit regulated by a suitable clock.   The temperature and rate of flow of air through the generator are regulated. For further details, consult the patent.

Mackin Process.—Mackin (1947) patented a process and equipment for producing high-grade vinegar without the use of shavings or a similar support.   The conditions of production may be accurately and continuously maintained.   Essentially the process consists of spraying a nutrient solution of dilute alcohol containing vinegar bacteria through the jet nozzles of a sparger located near the top of a confined space, allowing the mixture to fall as a fine mist through air being turbulently circulated in a countercurrent direction within the space, collecting the mixture near the bottom of the confined space, cooling it to below 95°F., and returning it for reatomizing.   The air used in the process is filtered before use.   The temperature is maintained between 68 and 95°F. by means of cooling water.   For further details of the process and for a description of the equipment, the reader is referred to U.S. Patent 2,423,897, July 15, 1947.

[1] ELMER, L. S., Vinegar Generator System, U S Patent 2,156,428, 1939

Revolving Generators.—Some vinegar is made by revolving generators.[1] These generators are essentially **rotating drums** or cylinders filled with shavings. The acidified nutrient alcoholic substrate is added in such quantity that the generator is approximately half filled (some drums hold 500 gal). The cylinder is then caused to rotate slowly, possibly at 1 5 r.p h., until vinegar of the desired acidity has been obtained, a process that may require about 3 weeks in some instances. Air, admitted through inlets, supplies oxygen to the upper portion of the drum The slow rate of rotation causes the mix to become oxygenated. This method, according to Cruess, is not too popular, probably on account of the expense involved in constructing the drums and in operating them, also owing to their complexity.[1]

Acetic Acid.—In one commercial method for producing acetic acid, ethyl alcohol is converted in large wooden tanks, or generators, to a fairly pure dilute solution of acetic acid. Dilute alcohol is permitted to flow from a small wooden tub situated on top of the tank to a revolving arm, located just under the cover of the tank, which distributes it over beechwood shavings that have been impregnated with acetic acid bacteria. The solution trickles slowly down through the generator, through the bottom of which air enters. The acid is subsequently used in the production of acetate esters

Crude acetic acid, or "pyroligneous acid," which is the principal source of commercial acid and acetates, is produced by dry distillation of certain kinds of hardwoods Since it has no microbiological implications it need not be considered here

Causes of Spoilage in the Vinegar Factory. *Vinegar Eels*—Vinegar eels, *i.e.*, nematode worms (*Anguillula aceti*), may be a source of considerable trouble in vinegar factories, especially when the fruit from which the cider or wine is made has not been carefully controlled. They also gain access from dirt brought into the plant, and from insects. They may attack the bacterial film and cause it to sink and in some instances cause deterioration of the vinegar. They are harmless to human beings but from an aesthetic or quality standpoint are very objectionable in a product Although quite small, about ¹⁄₈ in. long, they can readily be seen in a glass container by holding it before a strong source of light. In the factory they may be found around the edges of the surfaces of the liquid in barrels and in the generators. Their entrance can usually be prevented by keeping the plant in a high degree of cleanliness Empty casks may be sulphured lightly to prevent their access. Once in the vinegar they can be destroyed by heating the vinegar to a temperature of

[1] Cruess, W V , "Commercial Fruit and Vegetable Products," 3d ed , McGraw-Hill Book Company, Inc , New York, 1948

about 130°F. (54°C.) or by pasteurization, and they can be eliminated by filtration, using Filter-Cel, or by fining. *Infected barrels, tanks or generators may be treated with live steam.*

Mites.—Mites breed rapidly in the presence of warmth and moisture. Cleanliness of a high order may be necessary to prevent mites from appearing in an establishment. By placing a ring of turpentine or some other viscid or repellent substance around each air hole in a cask or barrel, their access may be prevented. In order to eliminate mites the methods used against eels may be employed. The room in which they are found must be thoroughly cleaned and may be washed with an emulsion of kerosene and water. Fumigating the room with sulphur helps sometimes. Steam and hot water will destroy the mites.

Vinegar Flies.—Vinegar flies (species of *Drosophila*) breed in decayed fruit, fruit juices, and vinegar. By preventing these substances from being spilled about and by keeping the factory scrupulously clean their presence can usually be avoided. The placing of screens over the windows and doors of the establishment and the use of fine screens over the holes in barrels in which the fermentation is taking place are very helpful in keeping out these and other flies.

Wine Flowers.—"Wine flowers" is the term used by wine manufacturers to denote the whitish film, often much plicated, composed of yeast-like cells, which grows on the surface of wines or nutrient alcoholic solutions. This film is sometimes called *Mycoderma vini*. The organisms making up the film are strongly aerobic, grow very rapidly, and in the course of time will oxidize many of the carbon-containing constituents to carbon dioxide and water. Flavor and alcohol are thus destroyed, while the solution becomes cloudy. Wine flowers can be prevented by storing the alcoholic solution in completely filled and closed containers or tanks, or by adding 1 part of vinegar to 3 parts of the alcoholic solution.

Darkening of Vinegar—Darkening of vinegar may be caused by iron and tannin or by an oxidase. As little as 1 part of iron in 10,000 parts of vinegar may cause a darkening of the product owing to the formation of iron tannates, if tannin is present in sufficient quantity. Iron is usually dissolved by the vinegar by contact with some iron source, while tannin may be extracted from new casks, especially oak, and also occurs in small quantity in the fruit juices. Aeration followed by fining satisfactorily removes the darkening caused by iron and tannin. The special type of oxidase, an oxidizing enzyme which produces darkening in fruit juices, may be destroyed by pasteurizing the vinegar.

Yields.—Under favorable conditions 50 to 55 parts of acetic acid may be obtained from 100 parts of sugar, or approximately 1.26 g. of acetic acid from 1 g. of ethanol, according to Cruess. A portion of the sugar is

consumed in the production of substances other than ethanol and as food by the yeast. There is also a loss usually of some alcohol and acetic acid by evaporation during the two fermentations.

Problem.—What yield of acetic acid can be obtained from 1 kg. of glucose, assuming 90 per cent efficiency in each of the conversion processes? How many grams and what volume (in liters) of air are theoretically required to convert the ethanol to acetic acid?

Solution

$$1 \quad \underset{\text{Glucose}}{\overset{(180)}{C_6H_{12}O_6}} = \underset{\text{Ethanol}}{\overset{(2 \times 46)}{2C_2H_5OH}} + \underset{\text{Carbon dioxide}}{\overset{(2 \times 44)}{2CO_2}} \quad \text{(Gay-Lussac equation)}$$

$$2 \quad \underset{\text{Ethanol}}{\overset{(2 \times 46)}{2C_2H_5OH}} + \underset{\text{Oxygen}}{\overset{(2 \times 32)}{2O_2}} = \underset{\text{Acetic acid}}{\overset{(2 \times 60)}{2CH_3COOH}} + 2H_2O$$
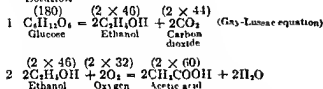
Theoretically, 1 mol of glucose (180 g.) will yield 2 mols, or 120 g., of acetic acid, which represents 2 parts acetic acid from 3 parts of glucose. One kilogram of glucose would yield (1,000/180) × 120 g. = 667 g. of acetic acid.

Assuming 90 per cent efficiency in both reactions (1) and (2), the yield of acetic acid would be 667 × 0.9 × 0.9 = 540 g. This represents a yield of 54 parts of acetic acid from 100 parts of glucose.

From Eq. (2), 92 g. of ethanol would require 64 g. of oxygen for conversion to acetic acid. Assuming 90 per cent efficiency in Eq. (1), 450 g. of ethanol [1000 × (92/180) × 0.9], would theoretically require (450/92) × 64 = 320 g. of oxygen = 224 liters. Since air is approximately one-fifth oxygen by volume, the quantity of air required to convert 1 kg. of glucose to acetic acid would be 1,600, or 1,120 liters. As a matter of fact not all the oxygen in the air becomes fixed by the bacteria, and several times this volume should be available.

Grains Strength.—The term "grains strength" is commonly used to express the concentration of the acetic acid in a vinegar. One-grain vinegar contains 0.1 g. of acetic acid in 100 cc. at 20°C. (1 mg. per cc.) Vinegar containing 1 g. of acetic acid per 100 cc. at 20°C., approximately 1 per cent, is of 10 grains strength. In other words, the grain strength is ten times the acetic acid content in percentage.

Uses of Vinegar.—Vinegar is used for several purposes in connection with food: as a condiment for direct use on the table or on a commercial scale in the manufacture of mayonnaise, French dressing, pickles, relishes, catsup, prepared mustard, and horse radish; for preventing mold growth in bread, and for other purposes. It may be used as an antiseptic.[1]

---

[1] McCulloch, E. C., "Disinfection and Sterilization," 2d ed., Lea & Febiger, Philadelphia, 1945.

Mechanism of the Acetic Acid Fermentation.—In the acetic acid fermentation, acetaldehyde is an established intermediate product, having been first shown to be such by Hoyer (1899). Its fixation can be demonstrated with neutral calcium sulphite.

Under anaerobic conditions 1 molecule of acetaldehyde may act as the hydrogen acceptor for a second hydrated molecule of the same substance. The result is a Cannizzaro reaction in which 1 molecule of ethanol and 1 molecule of acetic acid are produced from 2 molecules of acetaldehyde, the ethanol being formed by hydrogenation of acetaldehyde and the acetic acid by dehydrogenation of the hydrated acetaldehyde:

$$CH_3C\overset{O}{\underset{}{\diagup}}H + H_2O \rightarrow CH_3C\overset{OH}{\underset{OH}{\diagdown}}H$$

Acetaldehyde   Hydrated acetaldehyde

$$CH_3C\overset{OH}{\underset{H}{-OH}} + CH_3C\overset{O}{\underset{}{\diagup}}H \rightarrow CH_3C\overset{OH}{=}O + CH_3C\overset{OH}{\underset{H}{-}}H$$

Hydrated   Acetaldehyde   Acetic acid   Ethanol
acetaldehyde
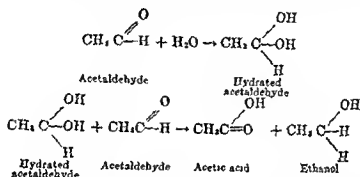
Neuberg and Windisch are of the opinion that ethanol is aerobically transformed to acetaldehyde, which in turn is dismutated, in the manner illustrated above, to equimolar quantities of ethanol and acetic acid. Alternate oxidation and dismutation follow until all the ethanol is converted to acetic acid.

Neuberg and Windisch[1] showed that *Acetobacter ascendens, A. pasteurianum* and *A. xylinum* were able to dismutate acetaldehyde to equimolar quantities of acetic acid and ethanol anaerobically. They likewise demonstrated that other aldehydes could be converted in a similar manner to their corresponding alcohols and acids.

Other workers[2] have shown that similar reactions may take place under anaerobic conditions.

It seems most likely[3] that in the normal acetic acid fermentation acetaldehyde is dehydrogenated to acetic acid. Oxygen acts as the hydrogen acceptor in the conversion of alcohol to acetaldehyde (a catalytic dehydrogenation):

[1] NEUBERG, C., und F WINDISCH, *Biochem Zeit.*, **166**: 454 (1925).

[2] WIELAND, H, und A. BERTHO, *Ann*, **467**: 98 (1928), MOLINARI, E, *Biochem. Zeit.*, **216**: 187 (1929)

[3] BUTLIN, K. R, "The Biochemical Activities of the Acetic Acid Bacteria," Chemistry Research, Special Report **2**, H. M Stationery Office, London, 1936.

$$CH_3\ C\!-\!H + O \rightarrow CH_3\ C\!=\!H + H_2O$$

Ethanol   Acetaldehyde
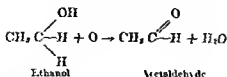
Acetaldehyde is hydrated:

$$CH_3\ C\!-\!H + H_2O \rightarrow CH_3\ C\!-\!OH$$

Acetaldehyde   Hydrated acetaldehyde
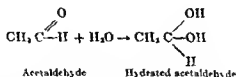
Two of the hydrogen atoms of the hydrated acetaldehyde are activated and donated to oxygen, the hydrogen acceptor·

$$CH_3\ C\!-\!OH + O \rightarrow CH_3\ C\!=\!O + H_2O$$

Hydrated acetaldehyde   Acetic acid
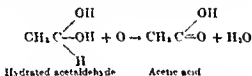
When conditions become favorable some acetaldehyde may be converted to acetic acid by dismutation   In this case acetaldehyde becomes the hydrogen acceptor *as well as* the donator

Substances other than oxygen and acetaldehyde may act as hydrogen acceptors, according to Wieland and Bertho[1]

Methylene blue and benzoquinone are two such substances

SOME OTHER FERMENTATIONS

The oxidations brought about by various species of the genus *Acetobacter* are of particular significance, since some of the compounds, such as ketoses and keto acids, formed from suitable substrates by these bacteria are prepared with considerable difficulty by purely chemical methods.

Species of the genus *Acetobacter* vary in their ability to oxidize or dehydrogenate various substances Some species, for example *A. rancens*, oxidize a substrate to a high degree, sometimes forming carbon dioxide and water as the principal end products. Such bacteria, obviously, have no industrial value   Other species of the genus *Acetobacter* bring about the incomplete oxidation of a substrate and, accordingly, may be of much importance

A. suboxydans is a species well-adapted for industrial use for it generally brings about the incomplete oxidation of sugars, alcohols, and acids even when a liberal supply of oxygen is available, as is essential for

[1] *Loc. cit*

a rapid dehydrogenation of the substrate. Butlin[1] and Kluyver and Boezaardt[2] have demonstrated, however, that the cells present in young cultures of *A. suboxydans* may produce some carbon dioxide from glucose

A. xylinum, the sorbose bacterium, produces incomplete oxidation of substrates also, but in the presence of large quantities of oxygen may oxidize the substrates completely, forming carbon dioxide and water.[2]

Methods of Production.—The production of oxidized products by the acetic acid bacteria, particularly *A. suboxydans*, may be carried out by surface culture or submerged culture methods. Illustrations of both procedures are presented on the following pages.

Fulmer and Underkofler (1947) reported that the optimum conditions for the production of polyhydric alcohols with *A. suboxydans* by surface culture methods were as follows: a pH of 6.1 (range of 5.1 to 6.8); a temperature of 28°C. (range of 25 to 30°C.); a yeast extract (Difco) concentration in the medium of 0.5 per cent; and a surface-volume (square centimeters per cubic centimeter of medium) ratio of about 1.195. They stated that sorbitol may be fermented in concentrations up to and including 35 per cent; mannitol, in concentrations up to and including 25 per cent; glycerol, in concentrations not exceeding 6 per cent; and erythritol, in concentrations not exceeding 4.5 per cent.

Nutrients of A. suboxydans.—The carbon is supplied as a polyhydric alcohol or other compound. Hydrolyzed casein or a known mixture of amino acids are satisfactory sources of organic nitrogen.[3] Mineral salts are necessary to supply the usual elements not found in the other ingredients. Pantothenic acid, *p*-aminobenzoic acid, and nicotinic acid are required as growth substances.[3]

Yeast extract or corn steep liquor are generally used to supply the growth requirements of *A. suboxydans*; hence it is not necessary to add growth substances, salts, or mixtures of amino acids. Fulmer, Bantz, and Underkofler (1944) found that an acid-alfalfa extract supported growth of *A. suboxydans* satisfactorily, but that it was somewhat inferior to yeast extract for this purpose. However, the yields of keto-compounds from glycerol, sorbitol, and 2,3-butylene glycol were of equal magnitude whether prepared with yeast extract or alfalfa extract.

Some Products Formed by A. suboxydans.—In Table 82 are shown some of the products formed by *A. suboxydans* as the result of the oxidation of suitable substrates. These products are in most instances also produced by other species of the genus *Acetobacter*.

[1] BUTLIN, K   :   · ‥   ) (1938)
[2] KLUYVER, A   · ‥   · · ·   Bact., 48: 183
[3] UNDERKOFLER, L. A., A. C. BANTZ, and ‥. ‥. ‥‥‥‥ ·
(1943).

TABLE 82.—SOME SUBSTRATES OXIDIZED BY *A. suboxydans* AND THE PRODUCTS FORMED

Substrate	Product formed	Substrate	Product formed
CH₂OH HOCH HOCH HCOH HCOH HCOH CH₂OH Perseitol	CH₂OH O=C HOCH HCOH HCOH HCOH CH₂OH Perseulose	CH₂OH HOCH HOCH HCOH HCOH CH₂OH d-Mannitol	CH₂OH O=C HOCH HCOH HCOH CH₂OH d-Fructose
CHO HCOH HOCH HCOH HCOH CH₂OH d-Glucose	COOH HCOH HOCH HCOH HCOH CH₂OH d-Gluconic acid	CH₂OH HCOH HCOH HCOH CH₂OH i-Adonitol	CH₂OH HCOH HCOH C=O CH₂OH "Adoninulose"
COOH HCOH HOCH HCOH HCOH CH₂OH d-Gluconic acid	COOH HCOH HOCH HCOH C=O CH₂OH d-5-Ketogluconic acid	CH₂OH HOCH HOCH CH₂OH Erythritol	CH₂OH O=C HOCH CH₂OH l-Erythrulose
COOH HCOH HOCH HCOH HCOH CH₂OH d-Gluconic acid	COOH C=O HOCH HCOH HCOH CH₂OH d-2-Ketogluconic acid	CH₃ HCOH HCOH CH₃ 2 3-Butylene glycol	CH₃ HCOH C=O CH₃ Acetylmethylcarbinol
CH₂OH HCOH HOCH HCOH HCOH CH₂OH d-Sorbitol	CH₂OH HCOH HOCH HCOH C=O CH₂OH l-Sorbose	CH₂OH HCOH CH₃ a-Propylene glycol	CH₂OH C=O CH₃ Acetol
		CH₂OH HCOH CH₂OH Glycerol	CH₂OH C=O CH₂OH Dihydroxyacetone
		CH₂OH CH₂OH Ethylene glycol	CH₂OH COOH Glycollic acid

The Sorbose Fermentation.—*l*-Sorbose is a compound of especial interest on account of its use in the synthesis of vitamin C. By chemical means, *d*-sorbitol is produced from dextrose; by biological dehydrogenation, *d*-sorbitol is converted to *l*-sorbose.

fermenters. Thus the time required for the conversion may have been reduced still further had the optimum pH been used.

The Production of Gluconic Acid.—The principal bacteria capable of producing gluconic acid in quantity from glucose belong to the group of acetic acid bacteria. Such acetic acid bacteria as *Acetobacter oxydans* may be used. These bacteria are able, under favorable conditions, to oxidize glucose to gluconic acid in the presence of an abundant supply of oxygen. Currie and Carter,[1] in a patent issued to them in 1933, suggested that glucose concentrations as high as 45 per cent may be used, but 25 per cent is stated to be the optimum concentration. From 0.2 to 2 per cent of mineral salts is added to the fermentation mash to satisfy the mineral requirements of the bacteria. The nutrient glucose solution is permitted to pass in a thin stream down through an apparatus similar to a vinegar generator. The presence of a large amount of oxygen is thus assured. A temperature of 15 to 35°C. is advocated for the fermentation. Hermann,[2] in an Austrian patent, suggested a similar plan for the production of gluconic acid.

Takahashi[3] (1934) advocated the use of *Bacterium Hoshigaki* var. *rosea* and *Bact. industrium* var. *Hoshigaki* in the fermentation of glucose or mannite solutions. Either soybeans or the extract of rice bran is added to the sugar solution to meet the nitrogen requirements of the fermentation organism. The fermentation is permitted to run for 18 days at 26 to 28°C. A very high yield is reported—as high as 103 per cent on the basis of the glucose.

The Production of 5-Ketogluconic Acid.—The structural formula of d-5-ketogluconic acid is as follows:

$$
\begin{array}{c}
COOH \\
| \\
HCOH \\
| \\
HOCH \\
| \\
HCOH \\
| \\
C=O \\
| \\
CH_2OH
\end{array}
$$

d-5-Ketogluconic acid
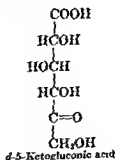
The literature concerning the formation of 5-ketogluconic acid has been reviewed by Stubbs, Lockwood, Roe, Tabenkin, and Ward (1940), who described the production of this acid and 2-ketogluconic acid. The information which follows is based on their report.

[1] U.S. Patent 1,896,811, 1933.
[2] Austrian Patent 133,139, 1933
[3] U S Patent 1,953,694, 1934

Organism Used.—A strain of *Acetobacter suboxydans* was used.

Preparation of Inoculum.—The inoculum was prepared in the following manner: *A. suboxydans* was grown for about 2 days at 30°C. in 10 ml. of a sterile medium containing 5 per cent sorbitol and 0.5 per cent yeast extract. This culture was employed to inoculate 200 ml. of a sterile medium containing 2 per cent glucose and 0.5 per cent yeast extract. The medium was aerated with 200 ml. of air per min. while in a Jena glass gas-washing bottle (type 101a). After about 2 days incubation at 30°C., this culture was used to inoculate 3 liters of a sterile medium containing 5 per cent glucose, 0.5 per cent yeast extract, and 37 g. of calcium carbonate in a 4-liter serological bottle. This medium was aerated with 5 liters of air per min. After about 2 days at 30°C., the inoculum was ready for use in seeding the main production medium.

Composition of Production Medium.—The production medium was prepared to contain the following components:

Components	Amount in Grams
Commercial glucose	118*
Corn steep liquor	5
Octadecyl alcohol[1]	0.3
Calcium carbonate[2]	27
Distilled water to make	1,000 ml.†

[1] Antifoam agent
[2] Sterilized separately
* Amount to provide 10 per cent glucose concentration
† The solution was sterilized by autoclaving at 20 lb. pressure for 20 min.

Conditions of Production.—The conditions of fermentation were as follows. A charge of 3 liters of the production medium was placed in a rotary drum of the type described in Chap. XXVI. The drum was rotated at 13 r.p.m., while air was introduced at the rate of 1,600 ml. per min. (measured as exit gas at atmospheric pressure), and the pressure in the drum was maintained at 30 lb. per sq. in. The contents of the drum was seeded with 300 ml. of inoculum, prepared as described above. The temperature was kept at 30°C.

Course of Fermentation.—In Fig. 66 is shown the course of a typical 5-ketogluconic acid fermentation.

Yields.—Yields of approximately 90 per cent of 5-ketogluconic acid were obtained in 33 hr. from 10 per cent glucose solutions.

Patented Process.—A patented process for the production of 5-ketogluconic acid has been described by Stubbs, Lockwood, and Ward (1913). A nutrient solution of glucose or calcium gluconate is acted upon by an active culture of *Acetobacter* under conditions of aeration and agitation at 25 to 30°C. The nutrients include corn steeping liquor; urea, amino acids, ammonium sulphate, or ammonium phosphate; potassium hydro-

gen phosphate; and magnesium sulphate. Calcium carbonate, zinc carbonate, or other compound are used as the neutralizing agents.

A - GLUCOSE IN SOLUTION
B - 5-KETOGLUCONIC ACID PRODUCTION
C - BACTERIAL POPULATION
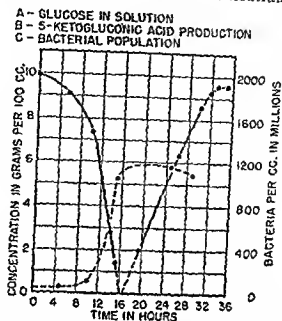
Fig. 66—Course of typical 5-ketogluconic acid fermentation. [*Courtesy of J. J. Stubbs, L. B. Lockwood, E. T. Roe, B. Tabenkin, and G. E. Ward, Ind. Eng. Chem., 32: 1626 (1940)*]

The Production of Acetylmethylcarbinol.—Acetylmethylcarbinol, known also as acetoin, has the following structural formula:

$$
\begin{array}{c}
CH_3 \\
| \\
HCOH \\
| \\
C=O \\
| \\
CH_3 \\
\text{Acetylmethylcarbinol}
\end{array}
$$
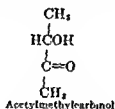
Its production in high yields from 2,3-butanediol has been described by Fulmer, Underkofler, and Bantz (1943); Underkofler, Fulmer, Bantz, and Kooi (1944); Sjolander and Eisenman (1946); and others.

Fulmer and his associates found that yields amounting to 90 to 94 per cent of the theoretical could be obtained from nutrient media containing the meso form of 2,3-butanediol. However, dextrorotatory 2,3-butanediol was not fermented. The following is an illustration of the procedure employed by Fulmer and his coworkers: Two and one-half liters of a medium containing 10 per cent glycol, 0.5 per cent yeast extract, and 0.5 per cent maltose and adjusted to an initial pH of 6.0 was sterilized in a 4-liter Erlenmeyer flask, cooled, and inoculated with 100 ml. of a 24-hr.-old culture of *Acetobacter suboxydans* (A.T.C.C. No. 621) grown in a butanediol-yeast extract-maltose medium. The medium was aerated during the conversion process.

Underkofler and his collaborators reported that only the meso and levorotatory forms of 2,3-butanediol were oxidized to acetylmethylcarbinol. Inasmuch as the 2,3-butanediol produced by *Aerobacter aerogenes* is largely of the meso form, the 2,3-butanediol in the fermented liquors (beers) from such a fermentation may be converted largely to acetoin.

Sjolander and Eisenman obtained 80 per cent conversion of the 2,3-butanediol in beers that contained in excess of 6 per cent of the glycol.

The Production of *l*-Erythrulose.—*l*-Erythrulose is formed by the oxidation of *meso*-erythritol in accordance with the following reaction:

$$
\begin{array}{ccc}
\text{CH}_2\text{OH} & & \text{CH}_2\text{OH} \\
\text{HO—C—H} & \xrightarrow{-\text{H}_2} & \text{C=O} \\
\text{HO—C—H} & & \text{HO—C—H} \\
\text{CH}_2\text{OH} & & \text{CH}_2\text{OH} \\
meso\text{-Erythritol} & & l\text{-Erythrulose}
\end{array}
$$
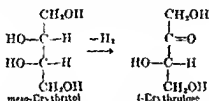
High yields of *l*-erythrulose have been obtained by Whistler and Underkofler (1938), who employed the following production method: The medium was prepared to contain 4.5 g. of *meso*-erythritol and 0.5 g of yeast extract per 100 ml. The pH was 6.1. The medium was distributed in 200-ml. amounts in 2-liter Erlenmeyer flasks and sterilized for 20 min at 15 lb steam pressure. The inoculum for a flask was 6 ml of a 24-hr. culture of *A. suboxydans* (A.T.C.C. No 621) grown in a medium of similar composition to the one described above. The inoculated medium was incubated for 9 days at 28°C. About 95 per cent of the *meso*-erythritol was converted to *l*-erythrulose under the foregoing conditions.

The Production of *d*-Tartaric Acid.—This acid, which has the following structural formula, may be produced in whole or in part by fermentation methods

$$
\begin{array}{c}
\text{COOH} \\
\text{HCOH} \\
\text{HOCH} \\
\text{COOH} \\
d\text{-Tartaric acid}
\end{array}
$$

Kamlet (1943) patented a process for producing *d*-tartaric acid from aerated aqueous solutions of glucose using *A. suboxydans* (A.T.C.C. No. 621) and a catalyst.

An example of the process follows: *A. suboxydans* is grown for 3 days on wort agar at 37°C. The culture is used to inoculate a liter of solution containing 10 per cent of glucose and 0.5 per cent yeast extract. This is aerated for 48 hr during incubation at 30°C and used to seed a substrate

Bertrand, G.: Préparation biochimique du sorbose, *Bull soc. chim*, (3)15: 627–631 (1896).

————: Sur le produit d'oxydation de la glycerine par le bactérie du sorbose, *Compt. rend*, 126: 842 (1898)

————: Préparation biochimique de la dioxyacetone cristallisée, *Compt. rend.*, 126: 984 (1898).

————: Sur la préparation biochimique de la dioxyacetone (propanediolone), *Bull. soc. chim.*, (3)19: 502 (1898).

————: Étude biochimique de la bactérie. *Ann. chim. phys.*, (8)3: 181–288 (1904).

Butlin, K. R.: The biological production of dihydroxyacetone, *Jour. Soc. Chem. Ind*, 57: 463 (1938).

Cozic, M.: "Étude biochimique de *Bacterium xylinum*," Thesis, André Lesot, Nemours, 1933.

Fischer, H. O. L., und H Mildbrand: Über Dioxyaceton, *Ber.*, 57: 707 (1924)

Kluyver, A. J, and F. J G. De Leeuw: *Acetobacter suboxydans* een merkwaardige azijnbacterie, *Tijdschr. Vergelijk Geneeskunde*, 10: 170 (1924).

Neuberg, C., und E. Hoffmann: Notiz über einfache Gewinnung von kristallisiertem Dioxyaceton auf biochemischem Wege, *Biochem. Zeit*, 279: 318 (1935)

Sazerac, R.: Sur une bactérie oxydante, son action sur l'alcool et la glycérine, *Compt rend* 137: 90 (1903).

Underkoflen, L. A., and E. I. Fulmer. The Production of Dihydroxyacetone by the Action of *Acetobacter suboxydans* upon Glycerol, *Jour. Am. Chem. Soc*, 59: 301 (1937).

Van Niel, C. B.: Biochemistry of Bacteria, *Ann. Rev. Biochem.*, 6: 595–620 (1937).

Virtanen, A I, und B. Barlund: Die Oxydation des Glycerins zu Dioxyaceton durch Bakterien, *Biochem Zeit*, 169: 169 (1926).

———— and M Nordlund: LXIV An Improved Method for the Preparation of Dihydroxyacetone, *Biochem Jour.*, 27: 442 (1933).

Some Patents

Currie, J N, and A. Finlay: Process of Fermentation, U.S Patent 1,908,225, 1933

British Patent 269,950, 1926.

Dutch Patent 27,804, 1932.

German Patent 563758, 1930.

Kamlet, J: Production of d-Tartaric Acid by Fermentation, U S Patent 2,314,831, Mar. 23, 1943

Pasternack, R, and E. V Brown· Preparation of D-Tartaric Acid, U S Patent 2,197,021, Apr 16, 1940

Siolander, N, and W. Eisenman: Process for Production of 3-Hydroxy-2-Butanone, U S. Patent 2,401,778, June 11, 1946.

Stubbs, J. J, L. B. Lockwood, E T Roe, and G. E Ward Fermentation Process for the Production of 5-Ketogluconic Acid, U S Patent 2,318,641, May 11, 1943.

General References on Biochemical Activities of Acetic Acid Bacteria

Bernhauer, K.: Biochemie der Essigbakterien, *Ergeb Enzymforsch.*, 7: 246–280 (1938)

————: "Gärungschemisches Praktikum," 2d ed., Verlag Julius Springer, Berlin, 1939

———— und B Görlich: Oxydationen mittels Essigbakterien IV Über die Bildung von 2-Ketogluconsaure durch *Bact gluconicum*, *Biochem Zeit*, 280: 367 (1935)

BUTLIN, K. R.: "The Biochemical Activities of the Acetic Acid Bacteria," Chemistry Research, Special Report 2, H. M. Stationery Office, London, 1936

————: Aerobic Breakdown of Glucose by *Bact. suboxydans, Biochem. Jour.,* 30: 1870 (1936).

————: LXVII. The Enzyme System of *Bact. Suboxydans* I Variation of Aerobic Activity with Age of Culture, *Biochem Jour,* 32: 508 (1938)

DUNNING, J W , E I FULMER, and L. A. UNDERKOFLER; The Oxidation of *i*-Inositol by the Action of *Acetobacter suboxydans, Iowa State Coll Jour Sci,* 15 (No 1): 39–46 (1940).

FULMER, E I , A C BANTZ, and L A UNDERKOFLER. The Use of Alfalfa Extract to Supply Nutrients for the Growth and Chemical Activities of *Acetobacter suboxydans, Iowa State Coll Jour Sci,* 18 (No 4), 369–376 (1944)

———— and L A UNDERKOFLER Oxidation of Polyhydric Alcohols by *Acetobacter suboxydans, Iowa State Coll Jour Sci,* 21 (No. 3): 251–270 (1947).

————, ————, and A C BANTZ The Production of Acetylmethylcarbinol by the Action of *Acetobacter suboxydans* upon 2,3-Butylene Glycol, *Jour Am Chem Soc,* 65: 1424–1427 (1943).

HANN, R M , E. B TILDEN, and C S HUDSON The Oxidation of Sugar Alcohols by *Acetobacter suboxydans, Jour Am Chem Soc,* 60: 1201 (1938)

HERMANN, S, und P NEUSCHUL Zur Biochemie der Essigbakterien, zugleich ein Vorschlag für eine neue Systematik, *Biochem Zeit,* 223: 129 (1931)

KLUYVER, A J , and A G J BOEZAARDT On the Oxidation of Glucose by *Acetobacter suboxydans, Rec trav chim,* 57: 609 (1938)

POSTERNAK, T VII. Sur la configuration de la meso-inosite, de la scyllite et d'un inosose obtenu par voie biochimique (scyllo-ms-inosose), *Helv Chim Acta,* 25: 746–752 (1942).

TILDEN, E B The Preparation of Perseulose by Oxidation of Perseitol with *Acetobacter suboxydans, Jour Bact,* 37. 629–637 (1939)

UNDERKOFLER, L A , A C BANTZ, and W H PETERSON: Growth Factors for Bacteria XIV Growth Requirements of *Acetobacter suboxydans, Jour Bact,* 45 (No 2) 183–190 (1943)

————, E I FULMER, A C BANTZ, and E R KOOI The Fermentation of the Stereoisomeric 2,3-Butanediols by *Acetobacter suboxydans, Iowa State Coll Jour Sci,* 18 (No 4) 377–379 (1944)

VAUGHN, R H. The Acetic Acid Bacteria, *Wallerstein Labs Communs,* 5 (No. 14): 5–26 (1942)

WATERMANN, H J Zur Physiologie der Essigbakterien *Centr Bakt, Parasitenk,* Abt II, 38: 451 (1913)

WHISTLER, R. L , and L A UNDERKOFLER: The Production of *l*-Erythrulose by the Action of *Acetobacter suboxydans* upon Erythritol, *Jour Am Chem. Soc.,* 60: 2507–2508 (1938)

CHAPTER XVI

THE PRODUCTION OF LACTIC ACID BY FERMENTATION

Lactic acid, or alpha-hydroxypropionic acid ($CH_3 \cdot CHOH \cdot COOH$) as an unnamed component of soured milk must have been known in human experience since the days when man first had his flocks and herds. Its true nature was discovered by Scheele, who isolated and identified it as the principal acid in sour milk in 1780.

Lactic acid was first discovered as a fermentation product by Blondeau in 1847. It was investigated by Pasteur as one of his first microbiological problems. Schultze (1868) demonstrated the presence of lactic acid bacteria in yeast cultures of distilleries. But it was not until the year 1877 that lactic acid bacteria were isolated in pure cultures, Dr. Lister having isolated *Streptococcus lactis*. During this same period Delbrück was endeavoring to determine the most favorable temperature for lactic acid fermentation in distilleries. He concluded that relatively high temperatures favored high yields of lactic acid.

Avery, of Littleton, Mass., was the first person (1881) to produce lactic acid successfully on a commercial basis. At that time an effort was being made to substitute calcium lactate for the tartrates then being used in baking powders. The attempt at replacement was unsuccessful but many new uses for the acid were found. Since 1881 the production of lactic acid by fermentation has become a very important industry. Lactic acid is now produced commercially from corn sugar, molasses, and whey.

Forms of Lactic Acid.—Lactic acid occurs in three forms: levorotatory lactic acid, dextrorotatory lactic acid (known also as "sarcolactic acid"), both of which are optically active acids, and *i*-lactic acid, an optically inactive acid

Lactic acid of various forms is produced by the lactic acid bacteria. *Lactobacillus delbrueckii* and *S lactis* usually produce *d*-lactic acid, while *L. leichmannii* and *Leuconostoc mesenteroides* var Sake commonly produce *l*-lactic acid. A few bacteria produce *i*-lactic acid, for example, *Lactobacillus pentoaceticus* (in "Bergey's Manual of Determinative Bacteriology"[1] this organism is listed as a probable synonym of *L. brevis*).

The lactic acid produced during fermentation is frequently inactive.

[1] 6th ed., The Williams & Wilkins Company, Baltimore, 1948.

Pederson[1] and others and later Tatum[2] and his associates showed that *Clostridium acetobutylicum* caused lactic acid bacteria, which usually formed active lactic acid, to produce inactive lactic acid. It was suggested by Tatum and his associates[2] that racemization might be brought about by an enzyme system elaborated by *Cl. acetobutylicum* and *Cl. butylicum*. It has been shown by Katagiri and Kitahara[4] that the enzyme racemase is responsible for the conversion of optically active lactic acids to inactive lactic acids. Thus the contaminants found in a fermentation medium may under certain circumstances be responsible for the racemization of active lactic acid.

Classification of Lactic Acid Bacteria.—A large number of bacteria produce lactic acid from carbohydrate materials. Many have no industrial significance, however.

In one method of classification the true lactic acid bacteria may be divided into two groups. One group is made up of those bacteria which convert carbohydrate materials to lactic acid as the principal end product. *L. delbrueckii* is an example of this group. Kluyver and Donker have suggested that this group be called the "homofermentative" lactic acid bacteria in contrast to a second group of lactic acid bacteria which produces, in addition to lactic acid, volatile acids and carbon dioxide in quantity, and for which they propose the name "heterofermentative." The latter group includes such bacteria as *L. lycopersici*, *L. mannitopoeus* and *L. acidophil-aerogenes*. Lactic and acetic acids, ethanol, glycerol, and carbon dioxide are the main end products formed by these bacteria.

In a second method of classification, the lactic-acid-forming bacteria may be grouped, according to their significance to man, into organisms of industrial importance, of which *L. delbrueckii*, *L. bulgaricus* and *Strept. lactis* are examples; organisms of possible therapeutic significance, such as *L. acidophilus*, and organisms of sanitary significance, such as *E. coli*.

THE COMMERCIAL PRODUCTION OF LACTIC ACID

Some General Considerations. *Organisms Used* —The organisms that may be used for the production of lactic acid by fermentation are *L. delbrueckii*, *L. casei*, *L. leichmannii*, *L. bulgaricus*, *L. pentosus*, and *Strept. lactis*. All these organisms are homofermentative.

[1] Pederson, C. S., W. H. Peterson, and E. B. Fred, *Jour. Biol. Chem.*, **68:** 151 (1926).
[2] Tatum, E. L., W. H. Peterson, and E. B. Fred, *Biochem. Jour.*, **26:** 846 (1932).
[3] *Ibid.*, **30:** 1892 (1936).
[4] Katagiri, H. and K. Kitahara. Refer to the papers cited at the end of the chapter.

Andersen and Werkman (1943) isolated and studied a sporeforming bacillus that produced large amounts of the dextrorotatory lactic acid. The name *Bacillus dextrolacticus* was proposed for this organism.

The type of organism selected for a fermentation depends primarily upon the carbohydrate being fermented and the temperature to be used. *L. bulgaricus*, *L. casei*, or *Strept. lactis*, may be used to ferment milk or whey, *L. bulgaricus* being favored. In the fermentation of dextrose or maltose *L. delbrueckii*, *L. leichmannii*, or *L. bulgaricus* may be used. Frequently *L. delbrueckii* is used with another lactic acid producer, such as *L. bulgaricus* or *Strept. lactis*, to ferment hydrolyzed starches.

Carbohydrates Suitable for Utilization.—A large number of carbohydrates may be utilized for lactic acid production. The acid is generally produced from glucose, sucrose, or lactose. Starches, corn and potato especially, may be hydrolyzed by enzymes, or by acids (preferably sulphuric acid) to maltose and glucose. Xylose is fermented by *L. pentoaceticus* to yield lactic acid and acetic acid, chiefly. Molasses and whey are generally low-priced sources of sugars for the fermentation. Smith and Claborn[1] have estimated that 2,700,000,000 lb. of lactose are obtained from skim milk, buttermilk, and whey annually. Of this quantity, a large percentage could be used in lactic acid production. Sulphite waste liquor and Jerusalem artichokes are potential sources of lactic acid. The choice of carbohydrate used will depend upon its availability; fermentability, with or without preliminary treatment; and its cost. In this country, corn sugar, molasses, and whey are much used; in Germany, potato starch.

Temperature of the Fermentation.—The lactic acid fermentation is carried out at comparatively high temperatures. In fermentations using *L. delbrueckii* a temperature of 45°C., or higher, may be maintained. *L. bulgaricus* may be incubated at 45 to 50°C.; *L. casei*, or *Strept. lactis*, at about 30°C. The optimum temperature should be determined experimentally for each type of fermentation.

Concentration of Sugar. . . . . concentration of 5 to 20 . . . . material and the conditions of the process.

Oxygen Relationship.—The bacteria used to produce lactic acid industrially are usually microaerophilic or anaerobic in nature. *Strept. lactis* is listed as a facultative aerobe.

pH.—The fermentation proceeds best when the pH is on the acid side of neutrality. Owing to the addition of calcium carbonate, calcium hydroxide, or some other neutralizing agent to the fermentation mash,

[1] SMITH, L. T., and H. V. CLABORN, *Ind. Eng. Chem.* (*News Ed.*), **17**: 370 (1939).

the pH tends to approach neutrality. The pH may be maintained at a constant value by the use of ammonia as the neutralizing agent.

Neutralization of Acid.—Lactic acid is neutralized by calcium hydroxide during fermentation in the following manner·

CH₃ CHOH—C
 OH
 HO
  + Ca →
 OH
 RO
CH₃ CHOH C
 O

Lactic acid Calcium hydroxide

CH₃ CHOH COO
    Ca + 2H₂O
CH₃ CHOH COO

Calcium lactate
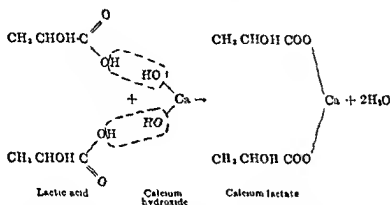
If the lactic acid were not neutralized, the lactic acid bacteria would not be able to tolerate the high acidity developed and the fermentation would not continue to completion.

Calcium (or zinc) hydroxide or carbonate may be added either at the beginning of the fermentation or intermittently as the fermentation progresses. Peterson, Fred, and Davenport suggested that the preliminary introduction of a neutralizing agent was as efficacious as intermittent introduction from the point of view of the speed and completeness of the conversion of xylose to lactic acid. The advantage of adding the carbonate intermittently lies in the fact that an acid reaction helps to prevent contaminants from gaining ascendency during the fermentation.

Growth Factors for Lactic Acid Bacteria.—Certain growth factors appear to be essential for certain lactic acid bacteria. Orla-Jensen[1] and his fellow workers reported that riboflavin and at least one other "activator" were required by certain lactic acid bacteria for normal development. Wood and his associates[2] confirmed their findings in respect to riboflavin.

Snell, Strong, and Peterson[3] described the preparation of an active

[1] Orla-Jensen, S., N. C. Otte, and A. Snog-Kjaer, *Centr. Bakt. Parasitenk.,* Abt. II, **94**: 434 (1936).

[2] Wood, H. G., A. A. Andersen, and C. H. Werkman, *Proc. Soc. Exptl. Biol. Med.,* **36**: 217-219 (1937).

[3] Snell, E. E., F. M. Strong, and W. H. Peterson, *Biochem. Jour.,* **31**: 1789-1799 (1937).

concentrate from liver extract, which was essential for the normal growth of the species of lactic acid bacteria investigated by them. It has been shown that this active substance was pantothenic acid.[1,2]

Nicotinic acid stimulated growth and acid production by some lactic acid bacteria.[1]

Lactobacillus pentosus 124-2 requires biotin, pantothenic acid, and nicotinic acid, according to Krueger and Peterson (1948).

For further information concerning this important subject, the reader is referred to the foregoing citations and others listed at the end of the chapter.

Accessory Nutrients in the Fermentation of Molasses.—Stiles and Pruess[3] have shown that good yields of lactic acid and a short fermentation period result from the addition of such accessory nutrients as malt sprouts, steep water, and thin grain residue to blackstrap molasses. The yield and fermentation time depend on the kind, quantity, and combination of the accessory nutrients.[3] It was suggested that the accessory nutrients supplied soluble organic nitrogen and stimulatory substances that were useful biologically.

Duration of Fermentation.—A fermentation is usually complete in from 42 hr to 6 days

Yields—Yields of 90 per cent on the basis of the sugar fermented are not uncommon in controlled processes. Higher yields have been obtained occasionally Totum and Peterson[4] have reported a yield of 100 5 g of *d*-lactic acid from 100 g of glucose.

Grades of Lactic Acid.—There are at least four principal grades of lactic acid These include a crude or technical grade of 22, 44, and 80 per cent strengths, edible lactic acid of 50 and 80 per cent strengths, plastic grade lactic acid of 50 and 80 per cent strengths, and U.S.P. grade lactic acid of 75 and 85 per cent strengths.[5]

Standards may be set up by the consumers, the requirements depending on the use to be made of the lactic acid. Color, flavor, and odor are important, in addition to the ash content For example, lactic acid used in the manufacture of transparent phenolic resins must be of high purity The chloride, sulphate, and ash contents must be very low, especially the iron.

[1] SNELL, E. E., F. M. STRONG, and W. H. PETERSON, *Jour. Am. Chem. Soc*, 60: 2825 (1938).

[2] SNELL, E. E., F. M. STRONG, and W. H. PETERSON, *Jour. Bact*, 38: 293–307 (1939).

[3] STILES, H R., and L. M. PRUESS, *Jour. Bact.*, 36: 149–153 (1938).

[4] TATUM, E L., and W. H. PETERSON, *Ind. Eng Chem*, 27: 1493 (1935).

[5] PECKHAM, G. T., JR., *Chem. Eng. News*, 22 (No 6): 440 (1944).

Lactic Acid Production by Continuous Fermentation.—A procedure for producing lactic acid from the lactose of sweet whey on a semiplant basis by continuous fermentation was worked out by Whittier and Rogers.[1] The main equipment used in this procedure included a storage tank for whey; a lime reservoir and feeding device; an insulated and covered fermentation tank with facilities for maintaining a constant temperature of 43° ± 0 1°C. and for agitating the medium at a slow rate; a storage tank to receive the overflow from the fermentation tank, which should have a capacity for 25 per cent of the latter tank; a coagulation tank, which should have 50 to 100 per cent of the operating capacity of the fermentation tank, and which is connected by way of a filter press or centrifuge to an evaporator.[2]

For operation, the fermentation tank is filled with sweet whey at a temperature of 43°C. The mash is inoculated with a culture of lactobacilli, for example, *L bulgaricus* or *L. casei*, and sometimes with a yeast, which by reason of its associated growth accelerates the fermentation. The inoculated medium is agitated and maintained at 43°C. (as closely as possible) for the duration of the fermentation.

After the pH of the mash has dropped to 5 0, usually after approximately 12 hr., lime is fed into it to maintain its pH between 5 0 and 5 8, a range favorable to the lactic acid bacteria but inhibitory to the development of contaminating organisms under the conditions of the fermentation.

At the end of 24 hr, and thereafter every 12 hr, the lactose content of the whey is ascertained When the amount of lactose has become diminished to less than 1 per cent, usually 48 to 72 hr. after the mash has been inoculated, whey, which may be pretreated with lime to inhibit the development of bacteria, is introduced into the fermentation tank from the whey storage tank. The whey is introduced at such a rate that the volume fed during 24 hr. equals the volume of the fermentation tank.[3] The rate is then adjusted for the most efficient operation. In this connection, the lactose content of the overflowing whey should be determined

The fermented whey is boiled until the proteins are entirely coagulated If lactic acid is desired, rather than calcium lactate, sulphuric acid is added to precipitate the calcium and liberate free lactic acid. The coagulated protein and calcium sulphate are then separated by filtration from the lactic acid, which is concentrated in evaporators to the desired concentration.

[1] Whittier, E. O., and L. A. Rogers, *Ind Eng. Chem*, **23**: 532 (1931).
[2] *Ibid*
[3] *Ibid*

Lactic Acid from Whey.—Lactic acid has been produced commercially from lactose since the year 1936 at Norwich, New York, in a plant operated by the Sheffield By-Products Company (a subsidiary of the Sheffield Farms, which in turn is a subsidiary of the National Dairy Products Corporation of New York). The plant has a capacity for 10,000 lb. of 22 per cent lactic acid per day. Technical and U.S.P. grades of lactic acid, calcium lactate, and sodium lactate are manufactured from whey.[1]

The utilization of casein whey has always been a problem. It may be dried and used as a feed for cattle and poultry, or the lactose and albumin may be recovered from it. Now, it may be used in the commercial production of lactic acid.

Surplus milk and skim milk from other sources may be used in the manufacture of lactic acid. The cream is removed from the milk and the casein precipitated by the use of lactic acid or hydrochloric acid, the former acid being preferred, for it may be recovered subsequently. The whey, known as casein whey, which is the product left after the separation of the cream and casein from the milk, contains lactalbumin (a protein), approximately 4.6 per cent lactose, vitamin G, mineral salts, and water. *This medium serves as the nutrient substrate in the manufacture of lactic acid.*

The commercial process for the production of lactic acid from the lactose of whey is based on the researches of Rogers and his associates of the Bureau of Animal Industry, U.S. Department of Agriculture. Chappell and his associates of the Sheffield By-Products Company and others of the research laboratories of the National Dairy Products Corporation at Baltimore were largely responsible for making the process a commercial one. This process has been described by Olive[2] and Burton.[3] The descriptions that follow are based on the articles by Dr. L. V. Burton.

The Process in Brief.—Pasteurized whey is inoculated with a starter containing *L. bulgaricus.* During the fermentation the lactic acid produced from lactose is neutralized intermittently with lime. At the end of the fermentation, the lactalbumin is coagulated by heat. After permitting the coagulated lactalbumin to settle, the solution of calcium lactate is decanted off, filtered, treated with decolorizing carbon and filter aids, filtered, and evaporated. Crystallization follows. The crystals are washed, perhaps further purified, dried, and may be sold as calcium lactate or converted to lactic acid. Various procedures are followed in producing the different grades of lactates and lactic acid.

[1] Olive, T. R., *Chem. & Met. Eng.,* **43:** 480–483 (1936).
[2] *Ibid*
[3] Burton, L. V., *Food Ind.,* **9:** 571, 634 (1937).

Fig. 67.—Lactic acid from casein whey   (Courtesy of L. V. Burton, Food Industries)

Preparation of the Starter.—The starter is prepared as follows. A quart of sterilized skim milk is inoculated with a culture of *L. bulgaricus*, containing also a yeast that causes the fermentation to become more vigorous. After incubation for 24 hr. at a temperature of 43°C (110°F.), the contents of the bottle are placed in a 40-qt. jug containing pasteurized skim milk. The contents of the jug are dumped, after incubation at a temperature of 43°C. for 24 hr., into a glass-lined steel tank containing 500 gal. of pasteurized whey. Following incubation at 43°C. for 24 hr., this starter is added to the main fermentation tank (see Fig. 67).

The Fermentation Tank.—This tank is constructed of wood and has a capacity for 5,000 gal. of whey. In the bottom of the fermenter is a perforated brass pipe, through which steam may be introduced to warm the mash to the temperature desired for the fermentation or for the subsequent coagulation of lactalbumin. The tank is provided with a mechanical stirrer and a 4-in. brass dip pipe, which may be raised or lowered in order to decant the clear solution of calcium lactate, which separates after the coagulation of lactalbumin. Before use, the tank is cleaned, treated with a chemical agent to destroy microorganisms and subsequently rinsed with pure water. An outlet is located at the bottom of the tank for use in connection with its cleaning.

The Fermentation.—The inoculated whey is maintained at a temperature of 43°C. until the end of the fermentation, which is usually complete in about 42 hr. Lime, $Ca(OH)_2$, in the form of a slurry, is added to the fermenting mash every 6 hr. to keep the acidity of the mash below 0.6 per cent. By neutralizing the lactic acid with lime, the fermentation time is shorter and the yields are higher, for under these circumstances the bacteria are not inhibited by the acid that they produce. At the completion of fermentation, the mash is neutralized to 0.1 per cent lactic acid.

Filtration of the Fermented Mash.—The fermented medium, which is of a pale-green[1] color, is heated to 96°C. (205°F.) to coagulate the lactalbumin. The coagulated albumin is permitted to settle. Only a short time is necessary, however, for in about 10 min. the supernatant liquor may be decanted.

The supernatant liquor is withdrawn through the decanting pipe and forced, by means of a centrifugal pump, through a plate-and-frame type of filter press to a storage tank. Here it is treated with lime to adjust the alkalinity to 0.1 per cent. A filter aid, such as diatomaceous earth, and a decolorizing carbon (*Norit*) are added. The mixture is thoroughly agitated and then the contents are permitted to stand. After approximately 15 min. the clear supernatant is decanted off and pumped through a filter press. The sludge is discarded. The clear solution, pumped to a

[1] *Ibid.*, **9:** 571 (1937).

clean wooden tank, is acidified to 0 05 per cent acid with lactic acid.
Filter aid and decolorizing carbon are again added, settling is permitted,
and the supernatant liquor is decanted and filtered. Sludge from this
second treatment may be used to treat a fresh lot of calcium lactate
solution.

Evaporation, Crystallization, and Washing.—The filtrate, or crude
liquor, may be stored in a clean wooden tank (Fig. 67), or it may be
concentrated in an evaporator, under a vacuum of 25 in., to a concentra-
tion of 15°Bé and then pumped to crystallizing pans (equipped with
casters to facilitate their being moved about) located on a gallery above
the floor containing the evaporators The crystallizing vats each have a
capacity for 300 gal Each is provided with a water jacket and the
inner wall, which comes in contact with the calcium lactate, has a lining
of stainless steel. Cooling water is passed through the jackets of the
pans. Calcium lactate crystallizes out after 10 to 12 hr at 10 to 15.6°C.
(50 to 60°F.) The pans are pushed to chutes and the crystallized mate-
rial shoveled down them to the baskets of Hepworth centrifugals. The
baskets are spun. The mother liquor, which passes off, is reserved for
further purification treatment. The crystals of calcium lactate that
remain are washed with water, while the baskets are still spinning. The
wash water thus obtained is evaporated to 13 5°Bé. and recrystallized,
and the new crop of crystals is centrifuged and washed The wash water
is again evaporated, crystallization takes place, and the crystals are
washed, the wash water being discarded this time.

From this series of treatments, three sets of crystals have resulted.
These are combined, placed in a glass-lined tank, and dissolved in a
small amount of water at 66°C (150°F.). Norit and filter aid are mixed
with the crude calcium lactate solution, the Norit for improving the color,
and the filter aid for removing finely suspended particles during filtration.
The supernatant is pumped to a filter press, while the sludge is returned
for the second treatment of the crude liquor. The thus refined liquor
may be concentrated to 11 5°Bé. and then placed in vats for crystalliza-
tion. Crystallization proceeds slowly, with the result that the crystals
are purer. The crystals are washed, the washings being returned to the
crude liquor, and then may be dried in a tunnel dryer. Such crystals
constitute the U.S P. grade of calcium lactate. The washed crystals,
without drying, may be used in the manufacture of the best grades of
lactic acid.

Grades of Lactic Acid.—Commercial, edible, and water-white grades of
lactic acid are manufactured by the Sheffield By-Products Company.

LACTIC ACID OF COMMERCIAL GRADE.—This grade of lactic acid is
produced from the crude calcium lactate liquor obtained after the first

vacuum treatment (Fig. 67). This liquor, which has a concentration of 13.5°Bé., is pumped to a wooden tank. Decolorizing carbon and chemical agents for precipitating *iron and heavy metals* are added. The mix is made slightly acid with an electrolytic grade of sulphuric acid and pumped through a rubber pipe to the upper tank of a vacuum filter made of stoneware. *Calcium sulphate and other precipitated matter are removed.* The precipitates are washed while on the filter. The filter cake is discarded, the filtrate and washings constituting a crude lactic acid solution of approximately 22 per cent strength.

The crude lactic acid may be stored in a wooden tank or further purified at once. The next step in the purification process is to concentrate the crude acid to 22°Bé. (50 to 60 per cent lactic acid) in a stainless-steel vacuum evaporator. The concentrated acid is conveyed to the upper tank of a vacuum filter, where Norit is added, together with sufficient lime or sulphuric acid to produce exact combination of the calcium and sulphate. Chemical agents may be added to precipitate any heavy metals still present. The mix is filtered, and the filter cake washed and disposed of. The filtered lactic acid is pumped to glass-lined tanks and adjusted to the desired concentration. Lactic acids of 50 and 44 per cent concentrations are in popular demand. The acid is placed in wooden barrels for distribution.

LACTIC ACID OF EDIBLE GRADE.—*The starting material* for the manufacture of the edible grade of lactic acid is the washed, solid, crude calcium lactate that is obtained from the centrifuge after the first crystallization (Fig. 67). This crude calcium lactate is added to a small amount of hot water in a wooden tank to form a solution. Sufficient sulphuric acid is added to combine all the calcium and sulphate as calcium sulphate Finally decolorizing carbon is added, and the mix is agitated thoroughly. The precipitates are removed as a sludge by vacuum filtration The lactic acid is stored in wooden tanks and eventually concentrated to 50 to 60 per cent strength in stainless-steel evaporating pans.

The purification treatment is repeated. Calcium and sulphate are exactly balanced, and a decolorizing carbon and a reagent to precipitate heavy metals are added. The mix is vacuum filtered, and the cake is washed. In glass-lined tanks, the lactic acid of approximately 18°Bé. is diluted to 50 or 44 per cent strength and placed in wooden b ls. Such acid is water clear.

WATER-WHITE LACTIC ACID.—
used by the chemical industry
calcium lactate may be used for i
generally used, since the neces "
The purification process is '

edible-grade lactic acid. The calcium is exactly balanced with sulphate, decolorizing carbon, etc., are added, and the mix is vacuum filtered. The acid from the vacuum evaporating pan is of 18°Bé. strength or contains about 65 per cent of lactic acid.

This acid must be entirely freed of calcium An excess of sulphuric acid is used to precipitate the last traces of this substance The filtered acid should give no precipitate when tested with ammonium oxalate.

Barium hydroxide is added to remove the excess of sulphuric acid, although a very slight excess of sulphuric acid is desired in view of the fact that the water used for diluting the lactic acid may contain a slight amount of calcium. Thus the very slight excess of sulphuric acid will balance the calcium added by the dilution water. A final filtration removes all precipitates of barium sulphate and calcium sulphate. Should distilled water be used for diluting the water-white acid to its final strength, dilutions may be made after the final filtration

The lactic acid is placed in barrels for marketing.

THE PRODUCTION OF WHITE CALCIUM LACTATE

A process for producing a white calcium lactate has been developed by Daly, Walsh, and Needle [1] The special features of this process are the use of a nondenatured milk as the nutrient and the rapid drying of the calcium lactate produced.

Whole milk, buttermilk, or skim milk, in liquid or in dried form,[2] may be used as the nutrient, but the protein must not be denatured. Nondenatured milk favors a rapid fermentation, thus avoiding the formation of undesirable colors or end products, and imparts no color to the medium.

The rapid drying of calcium lactate prevents caramelization and other color changes.

Calcium lactate may be produced according to the following example. A typical mash may contain the following ingredients

	Pounds
Sugar (dextrose)	6,600
Milk powder	150
Diammonium acid phosphate	50
Calcium carbonate	4,600
Water to 7,000 gal	

The foregoing mash is inoculated with 300 gal of a 24-hr culture of *L delbrueckii* and incubated at a temperature of 49°C. (120°F) for 5 to

[1] U S Patent 2,143,359, Jan 10, 1939
[2] Ibid

7 days, at the end of which time the total reducing sugars are usually less than 0.1 per cent. The use of the high temperature favors *L. delbrueckii* and inhibits the development of many other types of microorganisms.

The fermented mash is heated to 82°C. (180°F.) and held at that temperature for sufficient time to destroy the microorganisms present. Enough lime is then added (100 to 200 lb.) to bring the pH of the mash to approximately 11. The mash is filtered hot, proteins and insoluble compounds being removed. The filtrate is adjusted to a pH of 6 to 7 with lactic acid, for this pH range favors the volatilization of undesired organic acids and tends to prevent the formation of undesired color during the subsequent treatment. Using a vegetable carbon, the calcium lactate liquor is decolorized to yield a solution of water-white color. The solution is concentrated quickly to 20 to 21°Bé. by exposing a shallow layer to a large heating surface. This partially concentrated liquor is next spray-dried in a chamber into which gases enter at a temperature of approximately 232°C. (450°F.) and leave at a lower temperature. (Other types of drying may be substituted, if the drying is effected rapidly to prevent discoloration.) The resultant powder, maintained at a low temperature in order to avoid discoloration, is conveyed to a cyclone where it may be separated into relatively fine and coarse particles.

The product obtained by the foregoing process is noncrystalline, glass-like and homogeneous, with a calcium lactate content, based on the total solids, of 98 to 99 per cent. The moisture content is 5 to 6 per cent; the protein usually about 0.5 per cent.

If lactic acid is desired instead of calcium lactate, the filtrate from the lime treatment is decolorized with vegetable carbon and filtered. A measured quantity of sulphuric acid is then added to the filtrate, and the precipitate of calcium sulphate is removed by filtration. The resultant filtrate is decolorized with vegetable carbon and then concentrated to the desired strength by evaporation.[1]

THE PRODUCTION OF DEXTROLACTIC ACID

Tatum and Peterson[2] have described a method for producing *d*-lactic acid on a small scale. Batches containing 18 liters of a medium consisting of 3 per cent cerelose and 3 per cent malt sprouts were sterilized in 20-liter Pyrex bottles. The medium was inoculated with a pure culture of an organism that produced *d*-lactic acid. The inoculated medium was incubated at the temperature most favorable for the organism used, 30 or 37°C. After the fermentation had been in progress for

[1] *Ibid*

[2] TATUM, E. L., and W. H. PETERSON, *Ind. Eng. Chem.*, **27**: 1493 (1935).

24 hr., an excess of sterile calcium carbonate was added. Each bottle was shaken frequently during the 6 to 10 days of incubation in order to neutralize the acid as it was formed. Samples of the acid were converted to zinc lactate for analyses. Table 87 gives data for some of the fermentations carried out by Tatum and Peterson.

TABLE 87.—LACTIC ACID PRODUCTION BY VARIOUS ORGANISMS[1]

Organism	Tempera-ture of incuba-tion of glucose, °C	Lactic acid formed per 540 g. of glucose, grams	Glucose converted to lactic acid, per cent	Analysis of zinc lactate		Form of lactic acid
				Water of crystalli-zation, per cent	Specific rotation,[2] $[\alpha]_D^{20}$	
Strept. lactis, R	30	495	91	12 52	−8 65	Dextro
Strept. lactis, R	30	525	97	13 23	−8 65	Dextro
L. casei	30	505	93	12 80	−8 22	Dextro
L. delbrueckii, 3	37	530	98	13 00	−8 22	Dextro
L. delbrueckii, 3	37	520	96	12 90	−8 18	Dextro
L. delbrueckii, 3	37	500	92	12 86	−8 60	Dextro

[1] TATUM, E. L., and W H PETERSON *Ind Eng Chem*, **27:** 1493 (1935).
[2] 4 per cent concentration.

LACTIC ACID FROM SULPHITE WASTE LIQUOR

The production of lactic acid from sulphite waste liquor has been thoroughly studied by Leonard, Peterson, and Johnson (1948). They found that the best method for preparing the sulphite waste liquor for fermentation was as follows. The liquor was steam-stripped to remove the sulphur dioxide. During this treatment the pH was reduced to about 1. Next the liquor was treated with calcium hydroxide at 35°C. in such manner that it required about 10 min to raise the pH from 4 to 8 5. The mixture was held at pH 8 5, the region of sulphite precipitation, for 20 to 30 min and then filtered. Carbon dioxide was used to reduce the pH of the filtered medium to 7 or lower.

Lactobacillus pentosus 121-2 was found to be superior to other bacteria for producing lactic acid from sulphite waste liquors. It was prepared for inoculation purposes by repeated transfers at 8-hr intervals in a medium containing 3 per cent of malt sprouts and 3 per cent of glucose.

The inoculum was prepared by growing *L. pentosus* for 8 hr. at 30°C. in a mash containing 8 per cent of malt sprouts and 5 per cent of molasses The mash was prepared by steeping the malt sprouts (for example, 16 to

20 per cent concentrations at 45°C. for 8 to 12 hr.), adding dilute molasses, heating to 100°C. for 10 min., and cooling to 30°C.

Leonard and his associates found that nutrients could not be sterilized in the presence of sulphite waste liquor, for the lignin substances precipitated and bound some of the nutritive materials with the result that lower yields of lactic acid were obtained. The nutrients for the sulphite waste liquor were, accordingly, supplied with the inoculum.

The sulphite liquor, prepared as described above, was inoculated with 10 per cent by volume of the 8-hr. inoculum. The pH, which was initially about 6.5, usually fell to about 5.6 in 2 to 3 hr. It was maintained at about this level by the automatic addition of a slurry of slaked lime or calcium carbonate. Incubations were carried out for 40 to 48 hr. at 30°C.

The lactic acid was recovered from the fermented liquor in the following manner: The malt sprouts were removed by screening. The liquor was then concentrated to approximately 40 per cent of solids (30 per cent of its initial volume). The pH of the concentrate was reduced to 2 by the addition of sulphuric acid and the precipitate of calcium sulphate formed removed by filtration or centrifuging. The concentrated residue was extracted with a solvent (amyl alcohols and isophorone were good) at 90°C. This extract was washed with water to remove the acids (lactic and acetic). The aqueous solution of the acids was concentrated and the acetic acid separated out by distillation. The final product contained 90 per cent of lactic acid, 6 per cent of impurities, and 4 per cent of water.

Leonard and his coworkers estimated that a mill that produced 100 tons of pulp daily could produce 9,000,000 lb. of lactic acid annually on the basis of a 300-day year and of the production of 300 lb. of lactic acid from the sulphite waste liquors corresponding to 1 ton of pulp. Actually about 285 lb. of lactic acid and 75 lb. of acetic acid may be recovered from 2,000 gal. of sulphite waste liquor, which corresponds to 1 ton of pulp, at 95 per cent recovery efficiency.

D-LACTIC ACID FROM JERUSALEM ARTICHOKES

The production of d-lactic acid from Jerusalem artichokes has been studied by Andersen and Greaves (1942). The tubers were washed and ground. Two parts of water were added. The mixture was hydrolyzed by adjusting the pH to 2.0 with sulphuric acid and heating for 1 hr. at 95°C. Complete hydrolysis occurred under these conditions with no appreciable destruction of the reducing sugars. The hydrolyzate was neutralized with calcium carbonate and filter-pressed through a cloth.

Nutrients, calcium carbonate, and water were added to the diluted hydrolyzate in the following optimum concentrations:

Artichoke hydrolyzate (diluted 1 + 2)	500 ml.
(NH₄)₂SO₄.	. . . .	3 0 g.
KH₂PO₄.	. . . .	0 5 g.
Glucose or levulose	. . . .	110 0 g.
CaCO₃	. . . .	75 0 g.
Water	. . . . .	430 ml.

The amount of sugar added was sufficient to produce an over-all sugar concentration of about 14 g. per 100 ml   The quality of the final product could be improved by adding glucose or levulose to the diluted hydrolyzate.

The foregoing medium, dispensed in 200-ml. portions in 500-ml Erlenmeyer flasks, or in 2,000-ml. portions in 3-liter Fernbach flasks, was sterilized either by boiling or by autoclaving at a pressure of 15 lb. per sq. in. for 15 min.   After cooling to 47 to 50°C., it was inoculated with 5 to 10 per cent by volume of a 24-hr. culture of *Bacillus dextrolacticus* Andersen and Werkman.

The fermentations were carried out at 47 to 50°C., with agitation and aeration. The latter was essential for rapid and complete fermentation of the sugar.   Yields of 92 to 94 per cent of d-lactic acid were obtained in 4 to 5 days from the 14 per cent nutrient sugar solution as described above.

ACCELERATION OF THE FERMENTATION

Pan, Peterson, and Johnson (1940) reported on a method for accelerating the lactic acid fermentation of glucose or molasses by the use of unheated malt sprouts as a nutrient for *Lactobacillus delbrueckii*.   The increased speed of fermentation was due to a heat-labile growth factor contained in the malt sprouts

The following example illustrates the method employed by Pan and his associates.   Sixty-four gallons (210 liters) of diluted molasses (12.6 per cent invert sugar) were mixed with 14 7 lb. (6 67 kg.) of malt sprouts and heated to 45°C. in an open copper tank of 90-gal. capacity, which was provided with heating coils   This mash was inoculated with 4 liters of a 24-hr. culture of *L. delbrueckii*, which had been grown in a steamed medium containing 3 per cent of malt sprouts and 10 per cent of molasses. Thirty-three pounds of calcium carbonate were then added   During the fermentation the mash was stirred occasionally and the temperature was maintained within the range of 44 to 46°C   The fermentation was complete in 24 hr   Data concerning this fermentation and one carried out with Puerto Rican blackstrap molasses, in which a mechanical agitator was used to aid in the neutralization and maintenance of a uniform temperature, are presented in Table 88.

TABLE 88.—DATA OF LARGE-SCALE FERMENTATIONS[1,2]

	Run 1	Run 2[a]
Molasses used, lb	120.2	100.0
Sugar content of molasses, per cent	55.9	59.5
Malt sprouts, lb	14.7	14.5
CaCO₃ added, lb	33.0	32.5
Total volume, gal	63.8	63.0
Duration of fermentation, hr	21	16
Initial sugar concn., g./100 cc	12.6	11.32
Final sugar concn., g./100 cc	1.10	1.46
Fermentation, per cent	91.3	87.0
Lactic acid, g./100 cc	11.0	9.35
Yield, per cent sugar fermented	95.7	95.0
Yield, per cent sugar in molasses	87.3	82.6
Sp. rotation[3] of Ca lactate (4 per cent), [α]ᴅ	+6.16	+6.06
Form of lactic acid	levo	levo

[1] PAN, S. C., W. H. PETERSON, and M. J. JOHNSON, *Ind. Eng. Chem.*, **32**: 709 (1940).
[2] *Lactobacillus delbruckii* q used; 44 to 46°C.
[3] Value of [α]ᴅ for calcium lactate is 6.13.
[a] Puerto Rican blackstrap molasses.

THE PURIFICATION OF LACTIC ACID

In the purification of lactic acid, the object is to remove all ingredients that constitute impurities. These may include unfermented sugar, nitrogenous substances, coloring matter, calcium or sodium sulphate, and other salts.

Several methods may be used in purifying lactic acid. Smith and Claborn[1] of the Bureau of Dairy Industry, U.S. Department of Agriculture, have briefly discussed six methods for accomplishing purification. One method is purification by the process of recrystallizing calcium lactate, followed by treatment with sulphuric acid to liberate lactic acid. This process was described in detail in a foregoing section that dealt with the production of lactic acid from whey. Olive[2] and Burton[3] have described this process.

A second method consists in converting calcium lactate to zinc lactate, which crystallizes more readily than other lactates.[4] The zinc lactate is purified by repeated crystallizations. By adding hydrogen sulphide to zinc lactate, lactic acid is liberated and zinc sulphide pre-

[1] SMITH, L. T., and H. V. CLABORN, *Ind. Eng. Chem.* (*News Ed.*), **17**: 641 (1939).
[2] OLIVE, *loc. cit.*
[3] BURTON, *loc. cit.*
[4] SMITH, L. T., and H. V. CLABORN, *Ind. Eng. Chem.* (*News Ed.*), **17**: 641 (1939).

cipitated. Animal charcoal is added to decolorize the mass, which is then filtered. The filtrate, which contains the lactic acid, is concentrated by evaporation *in vacuo.*

In another method, esters of lactic acid are prepared, purified, and subsequently hydrolyzed to liberate lactic acid in a pure form. Methanol (CH_3OH) is added to the lactate in the proportion of 10 to 20 mols of methanol to 1 mol of calcium lactate or 2 mols of sodium lactate, which, preferably, should be free from moisture. Any undissolved precipitates are removed by filtration. Sulphuric acid is added, which accomplishes two objectives, liberation of lactic acid and catalysis of the subsequent esterification. In order to esterify the lactic acid, the mixture is heated for 4 to 8 hr. at a refluxing temperature. The precipitated substances are removed by filtration, and the excess methanol by distillation at atmospheric pressure. Using a low temperature, the water and most of the methyl lactate are distilled under vacuum. After diluting the distillate in the proportion of 2 to 3 parts of distilled water to 1 part of distillate, it is slowly fractionated in a corrosion-resistant column at atmospheric pressure. After hydrolysis of the methyl lactate, the methanol is recovered and the liberated lactic acid concentrated by evaporation *in vacuo.* Smith and Claborn state that chemically pure lactic acid may be prepared most efficiently and economically by this method.

Lactic acid may be partially purified in another process by gently oxidizing the crude liquor, which contains the lactates or lactic acid. Various oxidizing agents have been used for this purpose: sodium or calcium hypochlorite, potassium permanganate, potassium chromate, nitric acid, hydrogen peroxide, chlorine gas, and ozone gas.[1]

Lactic acid may be extracted from its water solution by the use of various solvents, one of which is isopropyl ether. This process is said to be expensive and hazardous, on account of the inflammability of the ether and possibility of explosive peroxides being formed.

Fractional distillation has not been used successfully on a commercial scale.

A method for purifying lactic acid and preparing a methyl lactate directly from the crude aqueous acid has been described by Filachione and Fisher (1946). The vapor of methanol is passed through the aqueous lactic acid. The effluent vapors, which are a mixture of methanol, water, and methyl lactate, are condensed. The condensate may be distilled in order to recover the methyl lactate, or it may be hydrolyzed to obtain purified lactic acid. Through a similar use of other alcohols, other esters of lactic acid may be obtained

¹ Smith and Claborn, *loc. cit.*

USES OF LACTIC ACID

Lactic acid has many uses.[1][2] These include uses in connection with foods, fermentations, pharmaceuticals, and the chemical industries. As an acidulant, the edible grade of lactic acid is used in confectionery, extracts, fruit juices and essences, lemonades, pickles, sirups, and in other products. Lactic acid may be used in the curing of meat and in canned vegetable and fish products. It acts as a preservative and prevents putrefactive changes from taking place in sauerkraut and pickles. It is used to acidulate worts in the manufacture of beer, to adjust the pH of the brine in the manufacture of pickled green olives, and to inhibit the development of butyric acid bacteria in the manufacture of yeast. It is used in making sherbets and effervescent beverages.

In the chemical industries, lactic acid is used in the dyeing of silks and other textile goods, as a mordant in the printing of woolens, in the batting and plumping of leathers, in the deliming of hides, in vegetable tanning, and as a flux for soft solders. The water-white grade is used in the plastic industry.

The lactates also have important uses. Calcium lactate is used in baking powders, in bread, in the pharmaceutical trade and for other purposes. Iron lactate is used in pharmaceutical manufacture. Sodium lactate is employed to aid in the retention of moisture by such products as tobacco. Copper lactate is a very important agent in a new process for the electrolytic deposition of metals.

Uses have been found for derivatives of lactic acid. Ethers of lactic acid, which may be represented by the general formula CH_3CHOR-$COOH$ and which are insoluble in water, may be used as solvents, plasticizers, and mordants in the manufacture of inks, plastics, and lacquers. Esters of lactic acid, which have the general formula CH_3CHOH-$COOR$, may be used for many of the same purposes as the ethers. The higher esters, for example, the butyl, amyl, and lauryl esters, possess greater stability than the lower esters, are insoluble in water, and are therefore more desirable than the latter.

MECHANISM OF THE LACTIC ACID FERMENTATIONS

Homofermentative Bacteria.—It has been suggested that the initial stages in the lactic acid fermentation may be similar to those of the ethyl alcohol fermentation. Phosphates added to a mash accelerate the fermentation. The removal of coenzyme from lactic acid bacteria will

[1] SMITH and CLABORN, op. cit., **17**: 370 (1939)
[2] BURTON, op. cit., **9**: 631 (1937).
[3] SMITH and CLABORN, loc. cit.

retard or cause the fermentation to cease. *L. delbrueckii* has the ability to convert hexosediphosphate to methylglyoxal, likewise, to convert methylglyoxal quantitatively to racemic lactic acid [1]

Lactic acid may arise thus through methylglyoxal by a mechanism similar to that of the alcoholic fermentation (refer to Chap. V).

$$C_6H_{12}O_6 \rightarrow 2CH_3COCHO + 2H_2O \quad \text{(By mechanism similar to that of the alcoholic fermentation)}$$
$$\underset{\text{Methylglyoxal}}{CH_3CO\,CHO} + H_2O \quad \underset{\text{Glyoxalase}}{\longrightarrow} \quad \underset{\text{Lactic acid}}{CH_3\,CHOH\,COOH}$$

The total changes may be expressed by the following equation:

$$\underset{\text{Glucose}}{C_6H_{12}O_6} \rightarrow \underset{\text{Lactic acid}}{2CH_3\,CHOH\,COOH}$$

Heterofermentative Bacteria.—Some of the first researches concerning the mechanism of the formation of the final products by heterofermentative lactic acid bacteria were carried out by Gayon and Dubourg;[2] by Fred, Peterson, and Davenport,[3] by Peterson and Fred,[4] and by others.

Nelson and Werkman[5] carried out experiments with several heterofermentative bacteria and obtained data upon which they have based a scheme for the dissimilation of glucose. The medium used by these men contained 2 per cent glucose, 1 per cent peptone, 0.3 per cent yeast extract (Difco), 0.6 per cent K_2HPO_4, and 0.6 per cent KH_2PO_4, with a pH of 6.2. Of these constituents, (1) the glucose, (2) the peptone and yeast extract, and (3) the phosphates were sterilized separately for 20 min. under a steam pressure of 20 lb. The separately sterilized components were combined just before inoculation. The mashes were incubated under an atmosphere of oxygen-free nitrogen at 30°C. for a period of 3 weeks before being analyzed

The data of Table 89 illustrate the kinds and quantities of the products formed by *L. acidophil-aerogenes* and *L. lycopersici* from glucose under the experimental conditions just mentioned. (*L. acidophil-aerogenes* and *L. lycopersici* are listed as probable synonyms of *L. brevis* in the sixth edition of "Bergey's Manual of Determinative Bacteriology")

Nelson and Werkman have suggested a tentative scheme for the

[1] ANDERSON, C. G., "An Introduction to Bacteriological Chemistry," William Wood & Company, The Williams & Wilkins Company, Baltimore, 1938
[2] GAYON, U., et E. DUBOURG, *Ann. Inst. Pasteur*, **15:** 527-569 (1901)
[3] FRED, E. B., W. H. PETERSON, and A. DAVENPORT, *Jour. Biol. Chem.*, **42:** 175-189 (1920)
[4] PETERSON, W. H., and E. B. FRED, *Jour. Biol. Chem.*, **44:** 29-45 (1920)
[5] NELSON, M. E., and C. H. WERKMAN, *Jour. Bact.*, **30:** 547-557 (1935)

TABLE 89.—ANAEROBIC DISSIMILATION OF GLUCOSE BY L. acidophil-aerogenes AND L. lycopersici[1]

Products	L. acidophil-aerogenes, millimols per liter	L. lycopersici, millimols per liter			
		I	II	III	IV
Glucose fermented	88 9	80.0	92.1	102.2	112 2
Ethyl alcohol ...	39 5	63.0	69.6	74.1	78 9
Acetic acid	10 2	12.1	16.5	15.5	19.5
Carbon dioxide . .	43 9	64 3	80.0	81.0	95 1
Lactic acid . . .	108.2	65 2	67.6	83.1	89 7
Glycerol . . .	21 7	25.0	36.2	32 6	43 5
Succinic acid .	0 8	3 8			
Percentage of carbon recovered:					
With succinic acid	100 2	101.2			
Without succinic acid	100 0	101 0	101.9	99.0	103.0
Oxidation-reduction ratio	0 873	0.851	0 912	0.884	0.943

[1] NELSON, M. E., and C. H. WERKMAN, Jour Bact, 30: 547-557 (1935).

dissimilation of glucose by heterofermentative bacteria. This scheme is given below.

$$C_6H_{12}O_6$$
Glucose

Hexose phosphate*

$$CHO \qquad CH_2OH$$
$$CHOH + 2H \rightarrow CHOH$$
$$CH_2OH \qquad CH_2OH$$
Glyceraldehyde*   Glycerol

$$C_3H_4O_3*$$
(Hypothetical 3-carbon compound)

$$CH_3CHO + CO_2 + 2H \qquad CH_3 CHOH COOH$$
Acetaldehyde   Lactic acid
$$\downarrow + 2H$$
$$CH_3CH_2OH \qquad CH_3 CO COOH + 2H$$
Ethyl alcohol   Pyruvic acid
$$\downarrow + H_2O$$
$$CH_3COOH + CO_2 + 2H$$
Acetic acid
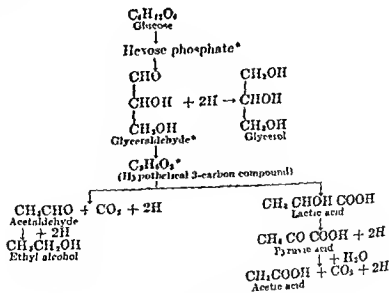
* These products have not been identified in the fermentation.

In the foregoing scheme, acetaldehyde and pyruvic acid are important intermediates. When acetaldehyde, or acetylmethylcarbinol, is added to a glucose fermentation, there is an increase in the quantities of acetic

acid and carbon dioxide, but a decrease in the quantities of ethanol, lactic acid, and glycerol formed from glucose.[1] Since both acetaldehyde and acetylmethylcarbinol are hydrogen acceptors, they will compete with the hydrogen acceptors, such as glyceraldehyde, which arise normally during the fermentation The result will be a decrease in the quantities of the compounds that are usually formed by reduction from these intermediary hydrogen acceptors.

The acetaldehyde added to a fermenting medium is reduced to ethanol.

Nelson and Werkman[2] have shown that pyruvic acid may be fermented by *L. lycopersici* with the production of equimolar amounts of acetic, lactic, and carbonic acids:

$$\underset{\text{Pyruvic acid}}{CH_3 CO\ COOH} + H_2O \rightarrow \underset{\substack{\text{OH} \\ \text{Hydrated pyru-} \\ \text{vic acid}}}{CH_3\ C\ COOH} \rightarrow \underset{\text{Acetic acid}}{CH_3COOH} + CO_2 + 2H$$

$$\underset{\text{Pyruvic acid}}{CH_3\ CO\ COOH} + 2H \rightarrow \underset{\substack{\text{OH} \\ \text{Lactic acid}}}{CH_3\ CHOH\ COOH}$$
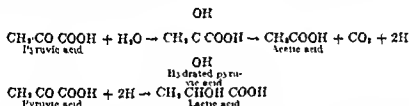
One molecule of pyruvic acid is hydrated to form 1 molecule each of acetic acid and carbon dioxide (carbonic acid), while a second molecule is reduced to a molecule of lactic acid

Mannitol Fermentation.—The fermentation, by lactic acid bacteria, in which mannitol is one of the products, has been studied by Boleato[3] and by Schoen and Eras[4] The mechanism of this fermentation will not be discussed here. For details in connection with this and the other types of fermentations discussed in the preceding paragraphs, the interested reader is urged to consult the references cited.

References on the Lactic Acid Fermentation

ANDREWS, A A, and J E. GREAVES *d*-Lactic Acid Fermentation of Jerusalem Artichokes, *Ind. Eng Chem*, **34**: 1522–1526 (1942).

ANDERSON, C G., "An Introduction to Bacteriological Chemistry," 2d ed, William Wood & Company, The Williams & Wilkins Company, Baltimore, 1946

BAETSLÉ, R : Preparation of lactic acid by fermentation, *Bull assoc. dixes inst super fermentations Gand*, **31**: 274–285 (1938).

BLONDEAU, C *Jour. Pharmacol*, (3) **12**: 244–261 (1847)

BOLCATO, V . Ricerche sui fermenti latto-mannitici II L'Acido latiico nella fermentazione mannitica del levulosio, *Ann chim applicata*, **26**: 24–30 (1936)

[1] *Ibid* , **31**: 603–610 (1936)

[2] NELSON, M. E., and C B WERKMAN, *Iowa State Coll Jour Sci*, **10**: 141–144 (1936)

[3] BOLCATO, V , *Ann. chim applicata*, **26** 21, 356, 423 (1936), *Enzymologia*, **5**: 52 (1938)

[4] SCHOEN, M, and E ERAS, *Enzymologia*, **4**: 198 (1937)

————: *Ricerche sui fermenti latto-mannitici. III. I prodotti della fermentazione del glucosio*, *Ann. chim. applicata*, **26**: 356–361 (1936).

————: *Ricerche sui fermenti lotto-mannitici. IV. L'influenza della reazione del mezzo sulla fermentazione del glucosio e del fruttosio*, *Ann. chim. applicata*, **26**: 423–427 (1936).

————: *Enzymologia*, **5**: 52 (1938).

BURTON, L. V.: Byproducts of Milk, *Food Inds.*, **8**: 571, 634 (1937).

DAVIS, J. G.: The nutritional requirements of the lactic acid bacteria, *Jour. Dairy Research*, **10**: 186–195 (1939).

ECKLES, C. H., W. B. COMBS, and H. MACY: "Milk and Milk Products," McGraw-Hill Book Company, Inc., New York, 1936.

FILACHIONE, E. M., and C. H. FISHER: Purification of Lactic Acid. Production of Methyl Lactate from Aqueous Solutions of Crude Acid, *Ind. Eng. Chem.*, **38**: 228–232 (1946).

FRIEDEMANN, T. E., M. COTONIO, and P. A. SHAFFER: Determination of Lactic Acid, *Jour. Biol. Chem.*, **73**: 335–358 (1927).

GARRETT, J. F.: Lactic Acid, *Ind. Eng. Chem.*, **22**: 1153–1154 (1930).

HAMMER, B. W.: "Dairy Bacteriology," 3d ed., John Wiley & Sons, Inc., New York, 1948.

KATAGIRI, H., and K. KITAHARA: On the Optical Properties of the Fermentation Lactic Acids. I. The Lactic Acid Bacteria Having the Special Nature of Causing Fermentation Lactic Acids to Form Inactive, *Bull. Agr. Chem. Soc. Japan*, **11**: 997–1007 (1935).

———— and ————: *II. The Manner of Lactic Acid Bacteria towards Inactive and Active Lactic Acids Added to the Fermentation Liquids*, *Bull. Agr. Chem. Soc. Japan*, **12**: 96–100 (1936).

———— and ————: *Part III. The Action of the Inactive Lactic Acid-producing Organisms upon the Optically Active Lactic Acids Added to the Fermentation Liquids*, *Bull. Agr. Chem. Soc. Japan*, **12**: 281–283 (1936).

———— and ————: *Part IV. A New Enzyme, Racemiase, Which Reveals Racemization of Lactic Acids*, *Bull. Agr. Chem. Soc. Japan*, **12**: 844–852 (1936).

———— and ————: *Part V. The Action of Acetone-Butyl Alcohol Producing Organisms upon Optically Active Lactic Acids*, *Bull. Agr. Chem. Soc. Japan*, **12**: 1217–1220 (1936).

———— and ————: *Part VI. On Some Properties of Racemiase*, *Bull. Agr. Chem. Soc. Japan*, **14**: 645–649 (1938).

———— and ————: *Racemiase, an Enzyme which Catalyses Racemization of Lactic Acids*, *Biochem. Jour.*, **31**: 909–914 (1937).

———— and ————: *CCXIII. The Lactic Dehydrogenase of Lactic Acid Bacteria*, *Biochem. Jour.*, **32**: 1654–1657 (1938).

———— and ————: *Studies on the Enzymes of Lactic Acid Bacteria. I. On Fumarase of Lactic Acid Bacteria*, *Bull. Agr. Chem. Soc. Japan*, **14**: 752–756 (1938).

KAYSER, M. E. Études sur la fermentation lactique, *Ann. Inst. Pasteur*, **8**: 737 (1894).

KOSER, S. A., and F. SAUNDERS: Accessory Growth Factors for Bacteria and Related Microorganisms, *Bact. Rev.*, **2**: 99–160 (1938).

KOZAI, Y.: Beitrage zur Kenntniss der spontanen Milchgewinnung, *Zeit. Hyg. Infektionskrankh.*, **31**: 337 (1899).

KRUEGER, K. K., and W. H. PETERSON: The Nutritional Requirements of *Lactobacillus pentosus* 124–2, *Jour. Bact.*, **55** (No. 5): 683–692 (1948).

LEONARD, R. H , W. H. PETERSON, and M. J JOHNSON: Lactic Acid from Fermentation of Sulfite Waste Liquor, *Ind Eng Chem*, **40**· 57-67 (1948)

LISTER, R.: *Quart Jour. Mic*, **18**: 177-178 (1878)

———: *Trans Path. Soc*, **29**: 425 (1878).

LUTMAN, B. F.: "Microbiology," McGraw-Hill Book Company, Inc., New York, 1929

MARSHALL, C. E.: "Microbiology," P. Blakiston's Son & Company, Philadelphia, 1921

MAY, O. E., and H. T HERRICK. Production of Organic Acids from Carbohydrates by Fermentation, *U.S Dept Agr*, *Circ* 216, May, 1932

MCCULLOCH, E C.: "Disinfection and Sterilization." 2d ed, Lea & Febiger, Philadelphia, 1945.

NELSON, M E, and C H. WERKMAN Dissimulation of Glucose by Heterofermentative Lactic Acid Bacteria, *Jour Bact*, **30**: 547-557 (1935)

——— and ———. Diversion of the Normal Heterolactic Dissimilation by Addition of Hydrogen Acceptors, *Jour Bact*, **31**: 603-610 (1936)

——— and ———: The Dissimilation of Pyruvic Acid by *Lactobacillus lycopersici*, *Iowa State Coll. Jour. Sci*, **10**: 141-144 (1936)

NENCKI, M.: Die isomeren Milchsäuren als Erkennungsmittel einzelner Spaltpilzarten, *Centr. Bakt. Parasitenk*, **9**: 304 (1891)

OLIVE, T. R : Waste Lactose Is Raw Material for a New Lactic Acid Process, *Chem. & Met. Eng*, **43**: 480-483 (1936)

ORLA-JENSEN, S.: The Lactic Acid Bacteria, *Kgl Danske Videnskab Selskab, Skrifter, Naturwidenskab Math. Aldo*, Ser **8, 5**. 81-196 (1919)

———: "Dairy Bacteriology," 2d ed (in English), P Blakiston's Son & Company, Philadelphia, 1931

———, N. C. OTTE, and A SNOO-KJAER Der Vitaminbedarf der Milchsäurebakterien, *Centr, Bakt Parasitenk*, Abt II, **94**: 431-447 (1936)

PAN, S C, W. H PETERSON, and M J JOHNSON Acceleration of Lactic Acid Fermentation by Heat-Labile Substances, *Ind Eng Chem*, **32**: 709-714 (1940)

PECKHAM, G. T, JR.· The Commercial Manufacture of Lactic Acid, *Chem Eng News*, **22**: 440-443, 469 (1944)

PEDERSON, C S : The Fermentation of Glucose, Fructose and Arabinose by Organisms from Spoiled Tomato Products, *N.Y State Agr Expt Sta*, *Tech Bull* 151, 1929

———: The Gas-producing Species of the Genus *Lactobacillus*, *Jour Bact*, **35**: 95-108 (1938)

———, W H PETERSON, and E. B. FRED The Forms of Lactic Acid Produced by Pure and Mixed Cultures of Bacteria, *Jour Biol Chem*, **68**: 151 (1926)

RODENKIRCHEN, J : Lactic acid fermentation and its causative organisms, *Forschungsdienst*, **6**: 163-200 (1938).

ROGERS, L. A. (associates of): "Fundamentals of Dairy Science," 2d ed, Reinhold Publishing Corporation, New York, 1936

SIMON, E.: Überführung von Milchsäure in Brenztraubensäure mittel Bakterien Delbrücki, *Biochem Zeit*, **245**: 488-493 (1932)

SMITH, L. T, and J CLABORN Utilization of Lactic Acid, *Ind Eng Chem (New Ed)*, **17**: 370 (1939)

——— and ——— The Production of Pure Lactic Acid, *Ind Eng Chem, (News Ed)*, **17**: 641 (1939).

SNELL, E. E, F. M. STRONG, and W. H. PETERSON: CCXXIII. Growth Factors for Bacteria. VI. Fractionation and Properties of an Accessory Factor for Lactic Acid Bacteria, *Biochem. Jour*, **31**: 1789-1799 (1937).

——, ——, and ——: Pantothenic and Nicotinic Acids as Growth Factors for Lactic Acid Bacteria, *Jour. Am. Chem. Soc.*, **60**: 2825 (1938).

——, ——, and ——: Growth Factors for Bacteria. VIII. Pantothenic and Nicotinic Acids as Essential Growth Factors for Lactic and Propionic Acid Bacteria, *Jour. Bact.*, **38**: 293-308 (1939).

——, E L TATUM, and W. H. PETERSON: Growth Factors for Bacteria. III. Some Nutritive Requirements of *Lactobacillus delbrückii*, *Jour. Bact*, **33**: 207-225 (1937)

STILES, H. R, and L M PRUESS. Nutrient Requirements of *L. delbrückii* in the Lactic Fermentation of Molasses, *Jour. Bact*, **35**: 149-153 (1938).

TATUM, E L, W H. PETERSON, and E. B. FRED: Effect of Associated Growth on Forms of Lactic Acid Produced by Certain Bacteria, *Biochem. Jour.*, **26**: 846-852 (1932)

—— and ——: Fermentation Method for Production of Dextro-Lactic Acid, *Ind Eng. Chem*, **27**: 1493 (1935)

——, ——, and E. B. FRED: Enzymic Racemization of Optically Active Lactic Acid, *Biochem. Jour.*, **30**: 1892-1897 (1936).

WHITTIER, E. O, and L. A. ROGERS: Continuous Fermentation in the Production of Lactic Acid, *Ind Eng Chem.*, **23**: 532-534 (1931).

WOOD, H. G, A A ANDERSEN, and C. H. WERKMAN: Growth-factors for Propionic and Lactic Acid Bacteria, *Proc. Soc. Expt Biol. Med.*, **36**: 217-219 (1937)

Some Patents

DALY, R. E, J F WALSH, and H. C. NEEDLE: Fermentation Process of Making White Calcium Lactate, U.S. Patent 2,143,359, Jan. 10, 1939.

FAITHFULL, S. E. Manufacture of Lactic Acid, U S. Patent 1,569,221, Jan 2, 1926

HAAG, I. L : Purification of Lactic Acid, U.S. Patent 2,013,096, Sept. 3, 1935

MACALLUM, A D.: Purification of Lactic Acid, U S Patent 2,013,104, Sept. 3, 1935.

NEEDLE, H. C : Fermentation Process for Producing Lactic Acid and Lactates, U S Patent 2,143,360, Jan. 10, 1939

PETERSON, W H, and E. B FRED: Production of Lactic and Acetic Acids, U.S. Patent 1,856,425, May 3, 1932.

STANDARD BRANDS, INC., U.S. Patent 2,032,433, 1936.

URBAIN, O M.: Process for Producing Lactic Acid, U S. Patent 2,009,753, July 30, 1935.

WALSH, J. F., and H C. NEEDLE: Lactic Acid Fermentation Process, U.S. Patent 2,143,358, Jan 10, 1939

CHAPTER XVII

SAUERKRAUT

Sauerkraut is the clean, sound

. . . product of characteristic flavors, obtained by full fermentation, chiefly lactic, of properly prepared and shredded cabbage in the presence of not less than 2 per cent, nor more than 3 per cent salt. It contains, upon completion of the fermentation, not less than 1.5 per cent of acid, expressed as lactic acid. Sauerkraut which has been rebrined in the process of canning or repacking contains not less than 1 per cent of acid, expressed as lactic acid.[1]

The fermentation is a natural one induced by bacteria resident on the leaves of the cabbage. By suitable control procedures involving selection and cleanliness of materials and regulation of the conditions of fermentation, an excellent food product is made.

Varieties of Cabbage Used for Sauerkraut Manufacture.—Sauerkraut may be manufactured from many different varieties of cabbage, but the varieties that grow slowly and form closely filled heads are preferred. All Seasons, All Head Early, Glory of Enkhuizen, and Flat Dutch are some of the desirable varieties.[2]

The variety grown will depend in part on local conditions of the climate and soil available.

Fully matured cabbage should be used for sauerkraut manufacture, since the use of green cabbage often leads to the production of kraut that is defective in color and in texture.[3]

Composition of Cabbage.—Various analyses have been given for cabbage.[4] Atwater and Bryant[5] state that cabbage contains 86 to 91.3 per cent water, 2.9 to 6.1 per cent sugar, 0.2 to 2.4 per cent protein, 0.1 to 0.7 per cent fat, 0.5 to 1.6 per cent fiber, and 0.1 to 2.1 per cent ash. Peterson, Fred, and Viljoen,[6] in analyses of fresh cabbage, found 91.0 to 93.9 per cent water, 3 to 4.2 per cent sugar, and 0.15 to 0.24 per cent total nitrogen.

[1] U S Dept of Agriculture, F D A, Service and Regulatory Announcements, Food and Drug, No 2, Rev 5, November, 1936

[2] LeFevre, E, U S Dept Agr, Circ 35, June, 1928

[3] Ibid

[4] Winton, A L, and K B Winton, "The Structure and Composition of Foods," Vol II, John Wiley & Sons, Inc, New York, 1935

[5] Atwater, W O, and A P Bryant, U S Dept Agr Bull 28, 1906

[6] Peterson, W H, E B Fred, and J A Viljoen, Canner, 61: 19 (1925).

Commercial Manufacturing Procedure.—Preparatory to shredding, sound cabbage is stored in well-ventilated buildings for at least 1 day to induce wilting and to obtain a product of more uniform temperature. The wilted cabbage should be cut with the production of the least amount of injury to the shreds, for damage might result in a subsequent softening of the sauerkraut. The spiral drill of a coring device cuts the core but does not remove it from the cabbage, which is passed on a conveyor to a trimming table where the outer leaves and any undesirable portions are removed. The cabbage is sliced by machine and the shreds are transported to the fermentation vats, some of which have a capacity of at least 80 tons of sauerkraut. Salt may be applied evenly to the shredded cabbage as it is being distributed in the vat or it may be spread through the product in the carts that carry the cabbage from the cutting rooms to the vats. A round cover is placed over the salted cabbage in each vat in such a manner that very little of the cabbage is left exposed to the air. Weights, usually cement blocks with handles, are placed on the cover to force the cabbage under the juices extracted from the shredded and salted product. When the acid content has reached at least 1.5 per cent, a process that may require from 3 to 4 or more weeks, depending on the temperature, the fermentation is usually complete.

Products of Fermentation.—Juices containing the sugars and other soluble substances are withdrawn from the shredded cabbage by the action of the salt. The bacteria that bring about the characteristic sauerkraut fermentation act upon the sugars and break them down to lactic and acetic acids, mannitol, ethyl alcohol, carbon dioxide, and small amounts of other products. Esters are formed from alcohol and the organic acids.

Microorganisms.—Many types of microorganisms may be found on the cabbage. Pederson[1] of the New York State Agricultural Experiment Station classifies the more important microorganisms that may affect fermentation into three groups: one group contains those bacteria which bring about the normal sauerkraut fermentation; one group contains spoilage bacteria; and one group contains yeasts

Desirable Types.—The common lactic-acid-producing bacteria that produce a normal sauerkraut fermentation may be further divided into three types: gas-producing *cocci*, non-gas-producing rods, and gas-producing rods. These three types are the only ones to develop in the normal controlled fermentation.[2] During the first part of the sauerkraut fermentation, the gas-producing cocci, *Leuconostoc mesenteroides*, predominate, for they find the salt concentration of 2.5 per cent and the

[1] Pederson, C. S., *N.Y. State Agr. Expt. Sta.*, *Bull.* 595, 1931.

[2] Pederson, C. S., *N.Y. State Agr. Expt. Sta.*, *Tech Bull.* 168, 1930.

temperature of 70°F., or below, favorable. These cocci attack the sugars incompletely and produce lactic and acetic acids, ethanol, mannitol, and carbon dioxide. They produce the least amount of change in the protein and in the cellular structure of the sauerkraut When the acid content increases to 0.7 to 1.0 per cent, they are destroyed. During their development, esters, which impart flavor to the sauerkraut, are formed from the acids and alcohol produced by them

The two other types of lactic-acid-producing bacteria do not develop rapidly at first under the conditions of the fermentation, but they gradually gain ascendency and carry the fermentation to completion One of these types, the non-gas-producing rods, includes *Lactobacillus cucumeris* (a probable synonym of *L plantarum*) and *L plantarum*, which produce lactic acid in large quantities (refer to the section on homofermentative lactobacilli) from a portion of the unchanged sugar and the mannitol, the latter substance having been produced from sugar by the gas-producing cocci. These lactobacilli are slowly destroyed, owing to their lack of resistance to the acid produced

Fermentation is completed by the third type, *L. pentoaceticus* (a probable synonym of *L brevis*), which produces lactic and acetic acids, ethanol, mannitol, and carbon dioxide from the remaining sugars. This organism is quite resistant to acid, withstanding an acid content as high as 2.4 per cent.

The following table, from Pederson's researches, shows analyses of partially and completely fermented sauerkrauts.

TABLE 90 —ANALYSES OF PARTIALLY AND COMPLETELY FERMENTED KRAUTS[1]

Kraut no.	Total acid, per cent	Lactic acid, per cent	Acetic acid, per cent	Acetic acid/ lactic acid ratio	Alcohol, per cent	Mannitol, per cent	Sugars
			Partially Fermented Kraut				
108	0 85	0 535	0 210	0 39	0 44	0 58	Trace
109	0 98	0 615	0 237	0 38	0 32	0 54	Trace
111	0 91	0 515	0 283	0 55	0 36	0 42	Trace
113	0 88	0 530	0 234	0 41	0 32	0 39	Trace
			Completely Fermented Kraut				
109	1 67	1 268	0 268	0 21	0 62	Trace	None
111	1 78	1 301	0 318	0 24	0 47	Trace	None
113	1 67	1 183	0 321	0 27	0 65	Trace	None

[1] PEDERSON, C S N Y State Agr Expt Sta, Bull 595 1931

A marked increase in the quantity of lactic acid with but slight increases in the acetic acid and alcohol content indicate that the non-gas-producing lactobacilli have dominated during the last stages of the fermentation.

The spoilage types of bacteria are usually aerobic and attack the proteins, exert but little action toward the sugars, and fail to grow in acid media. In the normal fermentation this group fails to gain ascendency.

Yeasts grow in the presence of oxygen, attack the sugars and lactic acid, but rarely develop during the early part of the fermentation They may cause pink sauerkraut unless controlled.

Starters.—The use of starters is not advocated, although under certain circumstances good to excellent results have been secured through their use.

A starter may consist of a pure culture, such as *Leuconostoc mesenteroides* or *Streptococcus lactis;* of a mixed culture, containing those organisms normally found in the sauerkraut fermentations; or of sauerkraut juice.

Starters containing pure cultures of the cocci that predominate during the initial period of the fermentation, or kraut juice, with an acidity of 0.25 per cent or less, withdrawn during the early stage of a fermentation, may bring about a normal fermentation. The value of the use of such starters is questionable, according to Pederson.[1]

When the sauerkraut juice contains more than 0.30 per cent of acid, the development of *L. mesenteroides* is inhibited and the resultant kraut is of an inferior quality. The non-gas-producing rods predominate under these conditions.

Old sauerkraut juices, juices containing more than 0.30 per cent acid, and cultures in which the lactobacilli (*Lactobacillus cucumeris* and *L. pentoaceticus*) predominate usually produce sauerkrauts of a poorer quality. For these reasons, sauerkraut manufacturers are advised to wash thoroughly and steam any vats in which a sauerkraut fermentation is contemplated and in which a fermentation has just been completed

Three factors are of much importance in regulating the fermentation of cabbage: the concentration of the salt, the covering of the sauerkraut, and the temperature of fermentation.

Salt.—Salt serves several important functions in the sauerkraut fermentation. It draws the juices out of the cabbage; it favors a lactic acid fermentation, the acid of which checks putrefaction; it contributes to the flavor; and it has much to do with firmness of the final product. The greater the amount of salt used, the more effective will be the extraction of juices, and the firmer will be the shredded cabbage leaves.

[1] PEDERSON, C S., *N.Y. State Agr. Expt. Sta., Tech Bull.* 216, 1933.

But high concentrations of salt will inhibit the desirable fermenting bacteria to some extent. It becomes necessary, therefore, to select a concentration that will permit a normal fermentation and give a firm, high-grade sauerkraut. A salt concentration of 2 5 per cent is favored by many commercial sauerkraut manufacturers—2.5 lb. of salt per 100 lb. of cabbage.

Spoilage types are not inhibited to any great extent until a salt content of 5 to 7 per cent is used. A high concentration of salt, therefore, cannot be depended on to prevent spoilage This concentration of salt is, moreover, prohibitive for the sauerkraut fermentation.

The salts used in the fermentation should be of a known, pure grade free from calcium and magnesium salts and should be measured by weight rather than by volume, since densities vary for different salts. Salt should be thoroughly mixed with the shredded cabbage.

In 1916, Henneberg[1] advanced the opinion that salt is added to cabbage only to withdraw juice. Hof[2] (1935) concluded, as the result of experiments in which 2 5 per cent salt was added to cabbage in one case and water but no salt in the second case, that the most important reason for adding salt to cabbage was to promote the formation of juice The sauerkraut made without salt was much softer than that prepared with salt Gerson[3] states that sauerkraut may be made by adding water, without salt, to the cabbage. Such sauerkraut is very soft, however

The salt concentration of a sauerkraut may be determined by titrating a sample of the juice with 0 1 N silver nitrate, using potassium chromate as the indicator.

Covering the Sauerkraut.—The covering of sauerkraut during fermentation is necessary to exclude oxygen, which favors the development of yeasts and spoilage types of bacteria (See the section on sauerkraut spoilage.) Weights are placed on the cover in a vat to force the extracted juices over the uppermost layer of sauerkraut Muslin cloth is sometimes used to aid in maintaining moisture in the product at the top of the vat

Temperature.—The optimum temperature for the sauerkraut fermentation is believed to be 65 to 70°F (18 3 to 21 1°C), while the range of 60 to 70°F. (15.6 to 21 1°C)[4] is satisfactory Any temperatures below 60°F. (15.6°C.) retard the fermentation. As was stated earlier, *Leuconostoc mesenteroides* grows well at temperatures below 70°F. (21.1°C) but

[1] Henneberg, W., *Deut Essigind*, **20**, No 21–22 (1916).

[2] Hof, T., *Rec. trav. botan. néerland.*, **32**: 93 (1935)

[3] Gerson, M., "Meine Diät," p. 96, Ullstein, Berlin, 1930.

[4] Pederson, C. S , *N.Y. State Agr. Expt Sta , Bull* 595, 1931.

Parmele, H. B , E. B. Fred, W. H. Peterson, J E. McConkie, and W. E. Vaughn, *Jour Agr. Research*, **35**: 1021 (1927)

at 60°F. (15.6°C.) or below, the fermentation will usually cease after the production of about 1 per cent of acid, since the lactobacilli fail to develop.

Experience has shown that the use of high temperatures for the fermentation is also undesirable. The use of temperatures around 86°F. (30°C.) favors the development of the lactobacilli but retards the development of *Leuconostoc*, which is primarily a flavor-producing organism. In order to maintain cabbage at this temperature, artificial heating must be employed. If the building is heated to above 86°F. (30°C.), as would be necessary, spoilage is likely to be incurred.

Spoilage of Sauerkraut.—*Aside from purely aesthetic reasons the importance of maintaining a sauerkraut factory in a high state of cleanliness is emphasized by a study of the following causes of spoilage.*

Dark sauerkraut is not uncommon and may be caused by a number of factors. Exposure of the sauerkraut to air may produce a dark product. Too much salt in certain cases, the result of uneven distribution, causes darkening of sauerkraut ("burned" sauerkraut), since normal fermenters but not spoilage types of bacteria are inhibited by high salt concentrations. Darkened sauerkraut may be induced by the use of excessively high temperatures during fermentation, conditions that favor the development of abnormal types of bacteria. The presence of iron and tannin (from wood), or the surface rotting of sauerkraut may also produce a darkened product. When the fermenting product is improperly weighted and the juice fails to cover or moisten the surface, aerobic bacteria and yeasts are likely to produce a darkening of the sauerkraut at the surface owing to the faulty fermentation that they cause.

Pink sauerkraut is caused by certain kinds of yeast. The types found on sauerkraut develop only when air is present. Hence they are usually found in the juice at the surface of the sauerkraut, occasionally as a white scum, when not well covered, or in the air pockets formed between the sides of the vat and the shredded cabbage, especially when the salt and cabbage have been unevenly mixed. Sugar and, in some cases, acid may be attacked by them. They also produce a pigment that may vary from a pink to an intense red in color. In completely and properly fermented normal sauerkraut these organisms do not usually develop.

The growth of pink yeasts is favored by factors that inhibit the normal sequence of bacterial growth.[1] Thus a high temperature of fermentation, a salt concentration greater than normal, an increased acid content, and a cabbage of low nitrogen content[2] may favor the growth of the yeasts producing a pink or red color in sauerkraut.

[1] Pederson, C S, and C D Kelly, *Food Research*, 3 : 583 (1938).
[2] Peterson, W. H., H. B Parmele, and E. B Fred *Soil Sci* , 24 : 299 (1927).

Soft sauerkraut may be due to the use of too little salt, high temperatures, faulty fermentation, or exposure to the air. The use of insufficient salt may produce a soft sauerkraut because it fails to draw enough juice from the cabbage or because it permits the lactic-acid-producing rods to develop. A high temperature favors the growth of lactobacilli at the beginning of the fermentation rather than in their proper sequence, with the result that the structure of the cabbage leaf is broken down to some extent. In such cases, the cocci fail to develop properly. Improper cleaning of a vat that has been packed previously, combined with high temperatures, favors the predominance of those types of bacteria which are commonly found at the end of the fermentation. These bacteria have, as previously stated, a greater ability to weaken the structure of the cabbage. Softening of sauerkraut by bacteria or yeasts growing on the surface of a product that has undergone a normal fermentation is not common.

Slimy sauerkraut is caused usually by the rapid growth of certain strains of *Lactobacillus cucumeris* and *L. plantarum*, especially ot raised temperatures. Such sauerkraut is edible but of an objectionoble chorocter. The slime may sometimes be dissolved by canning or cooking.

Rotted sauerkraut moy be caused by molds, yeasts, bacteria, or, once in a while, fruit flies. A heavy muslin cloth placed over the surface of the shredded cabbage and pushed down the sides of the vat at the time of packing it may be of considerable value in preventing rot. The cover is placed directly over the cloth with enough weights on top of it to press down the cabbage without causing the juice to flow over the cloth. Since the surface of the fermenting sauerkraut is kept moist the growth of undesirable types of bacteria will be inhibited Flies will be excluded by the cloth.

Off flavors may result from changes in the normal sequence of bacterial development, or from too rapid a fermentation

A method for preventing the development of aerobic organisms during the sauerkraut fermentation has been patented by Harrison (1911) The fermentation vessel is covered with a gastight closure, equipped with a release valve. The carbon dioxide produced during fermentation drives out the air. After the fermentation is complete, an atmosphere of carbon dioxide may be maintained by adding the gas from a pressure tank.

Cabbage for Sauerkraut.—Table 91 gives information concerning the production of cabbage for sauerkraut.

An examination of this table indicates that New York produces more than one-third of all the cabbage used for sauerkraut manufacture, while Wisconsin produces more than one-fifth Approximately

three-fifths of the total quantity of cabbage is thus produced by New York and Wisconsin.

Ohio is the third largest producer of cabbage for sauerkraut manufacture.

Growers receive different prices per ton for cabbage used for sauerkraut manufacturer. In 1928, the average price received by growers was $9 54 per ton; in 1932, $4.11; in 1936, $13.06;[1] in 1945, $13.31; and in 1946, $13.29.[2]

Prices paid in different states varied considerably; for example, the average price received by growers in New York was $11.10 per ton in 1946, while that for growers in Illinois was $16.60.[2]

Other information of interest concerning the statistics of cabbage production for sauerkraut manufacture and the location of plants will be found in *Technical Bulletin* 646, published by the U.S. Department of Agriculture.[3]

TABLE 91.—CABBAGE FOR KRAUT: 1946 WITH COMPARISONS[1]

State	Acres				Production, tons			
	10-year average		1945	1946	10-year average		1945	1946
	1927–1936	1935–1944			1927–1936	1935–1944		
New York	6,280	6,530	9,200	8,300	58,700	63,800	99,300	129,000
Ohio	2,220	1,840	2,200	2,600	18,900	14,500	19,800	29,100
Indiana	1,410	1,310	800	600	8,600	7,500	5,600	4,500
Illinois	670	460	500	680	4,200	2,900	4,000	3,700
Michigan	1,310	900	400	300	9,800	6,300	4,400	3,700
Wisconsin	4,640	4,760	5,800	6,300	34,500	34,700	65,000	61,200
Minnesota	330	200	300	200	2,700	1,400	2,400	1,900
Colorado	330	250	280	240	3,600	2,600	3,100	2,800
Washington	310	330	400	370	2,700	3,400	3,700	4,100
Other states[2]	1,660	2,180	2,850	2,660	11,000	15,000	26,000	25,500
Total, all states	19,160	18,760	22,730	22,250	154,700	152,400	233,300	264,800

[1] U.S. Department of Agriculture, Agricultural Statistics, 1947, Washington, D C , 1948.

[2] "Other states" includes Florida, Iowa, Maryland, Missouri, New Jersey, North Carolina, Oregon, Pennsylvania, Tennessee, Texas, Utah, and Virginia.

[1] U.S. Department of Agriculture, Bureau of Agricultural Economics, December, 1936.

[2] U.S. Dept. of Agriculture, Agricultural Statistics, 1947, Washington, D C , 1948.

[3] SPANGLER, R. L , Marketing Commercial Cabbage, U.S. Dept. Agr., Tech. Bull. 646, October, 1938

References on Sauerkraut

Anon: Making and Canning Sauerkraut, *Food Inds*, 17 (No. 10) 94–95 (1945).

Atwater, W. O., and A. P. Bryant: The Chemical Composition of American Food Materials, *U.S. Dept. Agr.*, Bull. 28, 1906.

Bachmann, F. M.: Growth of *Clostridium botulinum* in Fermented Vegetables, *Jour. Infectious Diseases*, 34: 129 (1924).

Bitting, A. W.: "Appetizing," Trade Pressroom, San Francisco, 1937.

Brunkow, O. R., W. H. Peterson, and E. B. Fred: The Influence of Certain Factors upon the Chemical Composition of Sauerkraut, *Jour. Am. Chem. Soc.*, 43: 2244 (1921).

———, ———, and ———: A Study of the Influence of Inoculation upon the Fermentation of Sauerkraut, *Jour. Agr. Research*, 30: 955 (1925).

Conrad, E.: Bakteriologische und chemische Studien uber Sauerkrautgährung, *Arch. Hyg.*, 29: 56 (1897).

Fabian, F. W.: The Science of Making Sauerkraut, *Mich. Agr. Expt. Sta.*, *Quart. Bull.*, 9: 50 (1926).

Fred, E. B., and W. H. Peterson: The Production of Pink Sauerkraut by Yeasts, *Jour. Bact.*, 7: 257 (1932).

Gerson, M.: "Meine Diät," p. 96, Ullstein, Berlin, 1930.

Harrison, W. H.: U.S. Patent 2,315,814, Apr. 4, 1914.

Henneberg, W.: Zur Kenntnis der milchsäurebakterien, *Zeit. Spiritusind.*, 26: 226, 213, 255, 257, 270, 277, 288, 291, 302, 315, 329, 341, 313 (1903).

———: Das Sauerkraut, *Deut. Essigind.*, 20: 133 (1916).

Hof, T.: Investigations Concerning Bacterial Iafe in Strong Brines, *Rec. trac. botan. néerland.*, 34: 93–173 (1935).

Keipper, C. H.: Improving the Quality of Kraut, *Canner*, 72 (part 2): 71 (1931).

LeFevre, E.: Use of Pure Cultures as "Starters" in the Preparation of Sauerkraut, *Canner*, 48 (8, part 2): 176 (1919).

———: Sauerkraut Production Controlled by Heat with the Report of an Experiment, *Canner*, 50 (10, part 2): 161 (1920).

———: Bacteriology of Sauerkraut and Pickles, *Chem. Age* (N.Y.), 30: 24 (1922).

———: Standardized Methods in Kraut Production, *Canning Age*, pp. 224, 232, 237 (1925).

———: The Commercial Production of Sauerkraut, *U.S. Dept. Agr.*, Circ. 35, June, 1928.

Marshall, C. E.: "Microbiology," P. Blakiston's Son & Company, Philadelphia, 1921.

Martin, E. A., W. H. Peterson, E. B. Fred, and W. E. Vaughn: Relation of Temperature of Fermentation to Quality of Sauerkraut, *Jour. Agr. Research*, 39: 285 (1929).

McConkie, J. E.: Types of Spoilage in Canned Sauerkraut, *Canning Age*, p. 240 (1927).

Parmele, H. B., E. B. Fred, W. H. Peterson, J. E. McConkie and W. E. Vaughn: Relation of Temperature to Rate and Type of Fermentation and the Quality of Commercial Sauerkraut, *Jour. Agr. Research*, 35: 1021 (1927).

Peterson, C. S.: Floral Changes in the Fermentation of Sauerkraut, *N.Y. State Agr. Expt. Sta.*, Tech. Bull. 168, 1930.

———: Sauerkraut, *N.Y. State Agr. Expt. Sta.*, Bull. 595, 1931.

————: The Relation between **Temperature and the Rate** of Fermentation of Commercial Sauerkaurt, *N.Y. State Agr. Expt. Sta., Bull.* 614, 1932.

————: The Effect of Inoculation on the Quality, Chemical Composition, and Bacterial Flora of Sauerkraut, *N.Y. State Agr. Expt Sta., Tech. Bull.,* 216, 1933

————: Controlling the **Quality** of Kraut during Manufacture, *Food Inds,* 19: 778–780, 212 (1947).

———— and C. D. KELLY: The **Quality of Commercial Sauerkraut,** *N.Y. State Agr. Expt. Sta., Bull* 613, 1932

———— and ————: Development **of Pink Color** in Sauerkraut, *Food Research,* 3: 583 (1938).

————, G L. MACK, and W L. ATHAWES: **Vitamin C Content** of Sauerkraut, *Food Research,* 4: 31 (1939)

PETERSON, W H, and E. B. FRED: An Abnormal Fermentation of Sauerkraut, *Centr Bakt Parasitenk.,* Abt II, 58: 199 (1923).

————, ————, and J. A. VILJOEN: Variations in the Chemical Composition of Cabbage and Sauerkraut, *Canner,* 61 (4): 19 (1925).

————, H. B. PARMELE, and E. B FRED: Some of the Factors Which Influence the Composition of Cabbage and Their Relation to the Quality of Sauerkraut, *Soil Sci,* 24: 299 (1927).

PRESCOTT, S C., and B E. PROCTOR: "Food Technology," McGraw-Hill Book Company, Inc, New York, 1937.

PREUSS, L M, W H PETERSON, and E. B. FRED: Gas Production in the Making of Sauerkraut, *Ind. Eng. Chem,* 20: 1187 (1928)

ROUND, L. A., Kraut Investigations, *Canner,* 44: (9, part 2): 113 (1917).

SPANGLER, R L.: Marketing Commercial Cabbage, *U.S. Dept Agr., Tech. Bull.* 646, October, 1938.

WEHMER, C.: Die Sauerkrautgärung, *Centr. Bakt Parasitenk.,* Abt II, 10: 625 (1903)

————: Untersuchungen uber Sauerkrautgärung, *Centr. Bakt. Parasitenk,* Abt. II, 14: 682 (1905)

WINTON, A L, and K B WINTON: "The Structure and Composition of Foods," Vol. II, John Wiley & Sons, Inc., New York, 1935.

CHAPTER XVIII

PICKLES

Pickles are

. . . immature cucumbers properly prepared, without taking up any metallic compounds other than salt, and preserved in any kind of vinegar, with or without spices. Pickled onions, pickled beets, pickled beans, and other pickled vegetables are vegetables, prepared as described above, and conform in name to the vegetables used.[1]

Classification of Pickles.—Owing to modifications in methods of preparation, there are a large variety of pickles. However, these may be conveniently grouped as dill, sour, and sweet pickles, according to the classification prepared by Fabian and Switzer,[2] which follows:

I. Dill pickles
  A. Fermented dill pickles
    a. Genuine dill pickles
    b. Genuine Kosher dill pickles
    c. Overnight dill or fresh fermented dill pickles
    d. Overnight or fresh fermented Kosher dill pickles
    e. Polish dill pickles
  B. Unfermented dill pickles made directly from fresh cucumbers
    a. Fresh or pasteurized dill pickles
    b. Iceberg or quartered dill pickles
  C. Dill pickles made from salt stock
    a. Processed imitation or summer dill pickles
    b. Processed imitation or summer Kosher dill pickles
    c. Pasteurized processed dill pickles
    d. Pasteurized processed Kosher dill pickles
II. Sour pickles
  A. Sour pickles
    a. Plain sour pickles
    b. Sliced or hot sour pickles
  B. Mixed sour pickles
    a. Mixed unspiced
    b. Spiced or hot mixed pickles
    c. Mixed chutney
  C. Relish chowchow, etc.
    a. Chowchow

[1] U. S. *Dept. of Agriculture, Service and Regulatory Announcements, Food Drug Admin.*, No. 2, Rev. 5, November, 1936.

[2] Fabian, F. W., and R. G. Switzer, *Fruit Products Jour.*, 20 (No. 5): 136 (1941).

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————: The Relation between **Temperature** and the Rate of Fermentation of Commercial Sauerkaurt, *N.Y. State Agr. Expt. Sta., Bull.* 614, 1932.

————: The Effect of Inoculation on the **Quality,** Chemical Composition, and Bacterial Flora of Sauerkraut, *N.Y. State Agr. Expt. Sta., Tech. Bull.,* 216, 1933.

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———— and C. D. KELLY· The Quality of Commercial Sauerkraut, *N.Y. State Agr. Expt. Sta , Bull.* 613, 1932.

———— and ————: Development of Pink Color in Sauerkraut, *Food Research,* 3: 583 (1938)

————, G L MACK, and W L. ATHAWES: Vitamin C Content of Sauerkraut, *Food Research,* 4: 31 (1939)

PETERSON, W H, and E B. FRED. An Abnormal Fermentation of Sauerkraut, *Centr. Bakt Parasitenk.,* Abt II, 58: 199 (1923).

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————, H. B. PARMELE, and E. B. FRED: Some of the Factors Which Influence the Composition of Cabbage and Their Relation to the Quality of Sauerkraut, *Soil Sci.,* 24: 299 (1927).

PRESCOTT, S. C, and B E PROCTOR: "Food Technology," McGraw-Hill Book Company, Inc , New York, 1937.

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SPANGLER, R. L. Marketing Commercial Cabbage, *U S Dept Agr., Tech Bull* 646, October, 1938

WEHMER, C.. Die Sauerkrautgärung, *Centr. Bakt. Parasitenk.,* Abt. II, 10: 625 (1903)

————· Untersuchungen über Sauerkrautgärung, *Centr. Bakt. Parasitenk ,* Abt II, 14: 682 (1905)

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        b Iceberg or quartered dill pickles
    C Dill pickles made from salt stock
        a Processed imitation or summer dill pickles
        b Processed imitation or summer Kosher dill pickles
        c. Pasteurized processed dill pickles
        d. Pasteurized processed Kosher dill pickles
II Sour pickles
    A Sour pickles
        a Plain sour pickles
        b Sliced or hot sour pickles
    B Mixed sour pickles
        a Mixed unspiced
        b Spiced or hot mixed pickles
        c Mixed chutney
    C Relish, chowchow, etc.
        a Chowchow

[1] U S Dept of Agriculture, Service and Regulatory Announcements, Food Drug Admin, No 2, Rev 5, November, 1936
[2] FABIAN, F W, and B G SWITZER, Fruit Products Jour, 20 (No, 5): 136 (1941)

III. Sweet pickles
- A. Plain sweet pickles
  - a. Standard sweet pickles
  - b. Midget sweet pickles
  - c Burgherkins
  - d. Slices, chips or wafers
  - e. Candied chips
  - f. Sweet dill pickles
  - g. Bread and butter or old-fashioned pickles
  - h Peeled pickles
- B. Mixed sweet pickles
  - a. Plain mixed
  - b. Mustard pickles
  - c. Jamaica pickles
- C. Relish or chopped sweet pickles
  - a. Plain relish
  - b Spread relish
  - c. India relish
  - d. Piccalilli
  - e Fruit relish
  - f. Mexican relish
  - g. Vegetable relish

Although almost any cucumbers, free from disease, may be used to make pickles, it is customary to select certain varieties, such as Snow's Perfection, Chicago Pickling, or Boston Pickling, which are preferable on account of their size, shape, or keeping qualities.

Preliminary Operations.—The harvesting, inspection, grading, and usually the washing of cucumbers are preliminary steps in the manufacture of most kinds of pickles. The cucumbers are harvested at the proper stage of maturity, usually when rather small, green, and firm. They are inspected for defects and those unsuitable on account of moldiness or other form of deterioration are discarded, since mold growth, even in slight amounts, may produce marked off-flavors in the pickled product, especially if it is of the "bread-and-butter type," and decomposed cucumbers may infect pickling tanks.

Cucumbers may be graded for size on the basis of the number required to fill a 45-gal. cask. For example, if the cask holds 800 cucumbers, they are classed as large; if 1,200, medium; and if 3,000, small.[1]

Although cucumbers are not always washed prior to processing, it is essential to clean thoroughly those used in the manufacture of unfermented pickles. Soaking of the cucumbers in clean water aids in loosening the soil Thorough washing removes any adhering dirt and generally reduces the number of microorganisms.

Cucumbers used for the production of salt stock or dill pickles are

[1] JONES, I D., and J. L. ETCHELLS, *Food Inds* ,15(No 1): 62 (1943).

placed in special vats and covered with brine. The vats, which are usually constructed of cypress, fir, pine, or redwood, are generally 6 to 8 ft. in depth and up to 16 ft. in diameter, and hold from 85 to 1,600 or more bushels of cucumbers. They are usually located in the open but may be erected in buildings. They must be thoroughly cleaned before use; and they may be soaked, prior to washing, either in water containing a minimum of 50 p.p.m of chlorine or in some other suitable germicide which will not impart odor, color, or toxicity to the tank or cucumbers. A false top is placed over the cucumbers and the brine is added until the former is submerged.

Salting.—There are in general two methods for adding brine to cucumbers, although there are many variations of these methods, and although pickles may be dry salted. In one method, a low-salt curing process, the cucumbers are added to a tank partially filled with brine, which gives a salinometer reading (refer to page 457) of 30° (nearly 8 per cent sodium chloride). This concentration of salt is considered to be the lowest that can be used with safety under ordinary conditions Salt is then added in the proportion of 9 lb for each 100 lb. of cucumbers in the tank. Thereafter sufficient salt is added to raise the brine 3° salinometer each week until a final reading of 60° (15 9 per cent) is obtained. Fermentation and curing are rapid by this method, but there is greater danger of undesirable bacteria predominating and the pickles are not likely to be so firm as when a higher initial concentration of salt is used.

In the second procedure, a brine containing 10.6 per cent salt (40° salinometer) is used The cucumbers are placed in the brine, and salt is added in the proportion of 9 lb. per 100 lb. of cucumbers. The salt concentration of the brine is increased 2° salinometer each week until it reaches 50° and thereafter 1° each week until a final reading of 60° salinometer is obtained Fermentation and curing by this procedure are slower, but there is less danger of spoilage types of microorganisms predominating, and the pickles are firmer than by the low-salt curing method This procedure is especially desirable where temperatures are likely to be relatively high, as in the South It is also advantageous in cold regions where the higher initial concentration of salt prevents freezing of the contents of the tank.

It is essential that very hard waters should not be used for pickle brines The salt should be of a high quality and should contain less than 1 per cent of sodium, calcium, or magnesium carbonates or bicarbonates, for these salts will neutralize the acid produced by the lactic acid-producing bacteria and favor the development of proteolytic types

It is the practice with some picklers to add starters from actively fermenting tanks or other containers Such starters contain acid-

producing bacteria of a desirable type. These bacteria are accustomed to high concentrations of salt and under such circumstances the acidity of the brine will increase rapidly. Since cucumbers contain less sugar than sauerkraut and often possess more mixed types of microorganisms, the use of a starter is frequently highly desirable. Sugar to the extent of approximately 1 per cent may be added to favor desirable fermentation, especially if the pickles are deficient in sugar, according to LeFevre

Once the tank has been filled, the curing and fermentation should be carefully controlled. The pickles should be kept well covered by brine at all times by the use of false covers or heads, which are fastened in place. The strength of the brine should be checked daily. After a tank has been filled and salt has been added, it is usually desirable to pump the brine from the bottom of the tank to the top in order to insure a uniform concentration of the brine. Any scum, containing yeasts or molds, that forms on the surface of the brine should be removed periodically to prevent consumption of the acids formed by fermentation.

Influence of Salt.—Cucumbers contain about 90 per cent of water. When cucumbers are in a tank surrounded by salt in the form of brine, they lose water by osmosis. Dissolved in the water are sugar, soluble proteins, minerals, and other substances, which are used as food by the lactic acid bacteria and any other microorganisms present, provided the conditions are favorable.

The brine is diluted and weakened as the result of the water being withdrawn from the cucumbers. At the same time some of the salt is absorbed by the cucumbers. Under these circumstances the salt concentration of the brine may become so reduced that spoilage types of bacteria may grow and perhaps predominate unless more salt is added to maintain a fairly high concentration of the brine.

Fabian and his associates[1] studying the influence of salt on bacterial activity in the cucumber fermentation found that the bacteria present during the first 24 hr. were largely peptonizers. After this period there was a gradual diminution in the numbers of bacteria until the brine reached 50° salinometer. The numbers then became fairly constant. The peptonizers were gradually replaced by acid-producing bacteria They died out more rapidly in 40° brine than in 30° brine, although the number of acid-producing bacteria was greater and "reached a maximum" quicker in the 30° brine than in the 40° brine.

The curing and fermentation of cucumbers, a process that is influenced by the salt concentration of the brine, the temperature, the size of the cucumbers, and the control of the scums (molds, yeasts), may require

[1] FABIAN, F. W., C. S. BRYAN, and J. L. ETCHELLS, *Mich. State Coll. Agr. Expt. Sta., Tech. Bull.* 126, November, 1932.

6 to 9 weeks. During the process, the flavor, texture, and color of the cucumbers change. The cucumbers are converted from a pale green to an olive or dark green in color. The color change extends throughout the pickle. Thus, if the pickle is opaque or white internally, it is not properly cured. Lactic acid bacteria are responsible for these changes, while salt is the most important factor in controlling the fermentation.
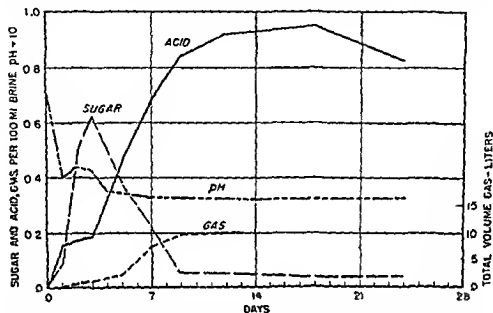
Fig. 68.4 — Relationship of changes in titratable brine acidity, brine sugar concentration and gas evolution from brine surface for a typical dill fermentation. (Acidity calculated as lactic acid, sugar concentration as reducing sugar.) *(Courtesy of J. D. Jones and J. L. Etchells, Food Inds., 15 (No. 1): 62-64 (1943).)*

Salt tends to preserve the chlorophyll and other constituents of the pickle.

Changes during the Cucumber Fermentation.—Complex physical, biochemical and microbiological changes take place during the fermentation of cucumbers. Some of the changes taking place have already been indicated; others are described in the following sections.

Biochemical Changes.—The physical and chemical changes taking place in the cucumber fermentations have been described by Jones and Etchells.[1] The fermentations observed were carried out during the summer months in vats of 85-bu capacity and of 6-ft diameter. These were erected outdoors and located in eastern North Carolina. The vats were filled with cucumbers and brine to a depth of 4½ ft. Observations were made in respect to brine sugar, acid, pH, and gases. Figure 68.4

[1] Jones and Etchells, loc. cit.
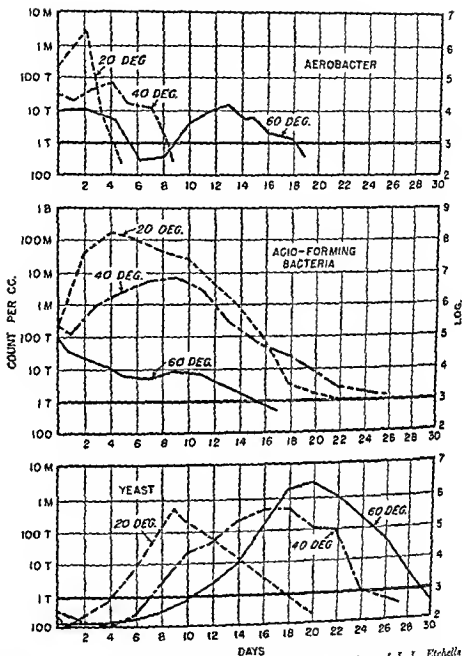
FIG 69 —Fermentation trends in 20, 40 and 60° brines. [Courtesy of J. L. Etchells and I. D. Jones, Food Inds, 15 (No 2): 54–56 (1943).]

cerning the microbiological populations of the brines at different initial salinities. Counts of microorganisms are plotted logarithmically.

As a result of their investigations, Etchells and Jones drew certain conclusions. The fermentation by the *Aerobacter* group usually occurs in the 60° brines, gas evolution being vigorous; it may or may not occur in the 40° brine; and it is brief in duration if it occurs in the 20° brine. The relatively weak *Aerobacter* fermentation in the 20° brine is attributed to

the rapid growth of the acid-formers which inhibit the development of the gas-producing bacteria. The acid-formers grow best in the 20° brine and poorest in the 60° brine. The combination of acid and salt appears to restrict the activities of this group. Active yeast fermentation takes place at all of the brine concentrations.

The influence of adding sugar to brines has been studied by Veldhuis and his associates.[1] They found that the addition of sugar to brines at the beginning of or during active fermentation resulted in an increase in the number of acid-formers, but if sugar were added towards the end of active fermentation, it produced no appreciable change. The addition of sugar to brine increased also the number of yeasts and the amount of gas evolved.[1]

A summary of information concerning the general relationships in fermentations due to species of *Aerobacter*, acid-forming bacteria, and yeasts in the 20, 40, and 60° brines is presented in the following table prepared by Etchells and Jones[2]

TABLE 93.—GENERAL RELATIONSHIPS IN FERMENTATIONS DUE TO *Aerobacter*, ACID-FORMING BACTERIA, AND YEASTS IN 20, 40 AND 60° BRINES[3]

Organisms	Brine conc., deg sal	General type of fermentation	Fermentation activity	Chief products formed	Approximate start of fermentation[1]	Approximate duration of fermentation
Aerobacter	20	Flash-gaseous	Slight, may be absent	Hydrogen and CO_2	1st day	2–3 days
	40	Gaseous	May or may not be active	Hydrogen and CO_2	1st–3d day	1 week
	60	Gaseous	Usually active	Hydrogen and CO_2	7th–8th day	1 week
Acid forming bacteria	20	Acid	Very active	Lactic acid	1st day	3 weeks
	40	Acid	Active	Lactic acid	2nd–3d day	3 weeks
	60	Acid	Very slight			
Yeasts	20	Gaseous	Active	CO_2 and alcohol	3d–5th day	2 weeks
	40	Gaseous	Active	CO_2 and alcohol	7th day	17 days
	60	Gaseous	Active	CO_2 and alcohol	11th–12th day	17–21 days

[1] ETCHELLS, J. L. and J. D. JONES, *Food Inds.* **16** (No. 2) 51 (1943).
[2] Brine temperature about 80° F. during the fermentation period.
[3] After bleeding tanks.

Microbiological studies concerning cucumber fermentations have been

[1] VELDHUIS, M. K., J. L. ETCHELLS, J. D. JONES, and O. VELDHUIS, *Food Inds.*, **13** (No. 10). 51 56, (No. 11) 48 50 (1941).
[2] ETCHELLS and JONES, *loc. cit.*

carried out by a number of other investigators. References to some of these studies are listed at the end of the chapter. The importance of yeasts has been demonstrated by Etchells[1] and by Jones and his associates[2] The *Aerobacter* fermentation has been studied by Etchells, Fabian, and Jones.[3]

Control of Scum.—Scum formation is caused commonly by film-forming yeasts (mycoderma), although molds may occasionally appear in scum on the surface of brine unless proper care is exercised. The organisms in the scum utilize the acid produced by the acid-formers and sugars, produce off-odors, and result in the production of inferior products.

Vats are usually erected outdoors, since scums are less likely to form when the surface of the brine is exposed to the action of sunlight However, such a location is not always advantageous, for rain may dilute the brine and dust may be a problem.

Some investigators have endeavored to control scum formation through the use of ultraviolet light. Rahn[4] found that the irradiation of the brine for 30 min. each day with ultraviolet light (from an Uviac lamp) at a distance of 6 ft. from the surface prevented the formation of scum. Fabian and Bryan[5] used a Cooper-Hewitt mercury-vapor ultraviolet lamp to irradiate mycoderma. They found that there was considerable variation in resistance to ultraviolet light exhibited by different strains of mycoderma, that young cultures were more resistant than old ones, and that it was difficult to destroy mycoderma when the scum was thick.

Etchells and Veldhuis[6] attempted to control the growth of mycoderma by the use of mineral oil. They discovered that the culture of mycoderma used by them was able to grow under a layer of the oil and utilized acid and dextrose from the brine. However, it appeared that the use of a light mineral oil resulted in a somewhat slower rate of the disappearance of the brine acid

Fabian[7] has demonstrated that scum formation on the surface of genuine dill pickle brine may be prevented by the addition of a few drops of mustard oil or by covering the surface with paper or some other suitable

[1] Etchells, J. L , *Food Research*, 6 (No. 1): 95 (1941)

[2] Jones, I D , M K. Veldhuis, J. L. Etchells, and O. Veerhoff, *Food Research*, 5 (No 5): 533 (1940).

[3] Etchells, L J , F. W. Fabian, and I. D. Jones, *Mich State Coll Agri Expt Sta* , *Tech Bull* 200, June, 1945.

[4] Rahn, O , *Canner*, pp. 42–43 (July, 1931).

[5] Fabian, F. W , and C S Bryan, *Fruit Products Jour.*, 11: 134 (1932).

[6] Etchells, J. L , and M K. Veldhuis, *Fruit Products Jour.*, 18 (No. 9): 265–267, 280 (1939)

[7] Fabian, F W , *Fruit Products Jour* , 19: 244 (1940).

material that has been previously impregnated with mustard oil. Later Blum and Fabian[1] studied the effect of 32 spice essential oils and seven components on the control of microbial surface growth. They found that oil of mustard was superior to the rest, after which followed cinnamon, cassia, and cloves.

Processing.—The amount of salt removed depends upon the use that is to be made of the pickles. Unless they are used as salt pickles, a large part, but not all, of the salt is removed.

The salt pickles are placed in tanks equipped with a supply of steam. The temperature is raised to 120°F. (49°C.), or above, and the pickles agitated by means of paddles or compressed air. The water may be changed and the process repeated until the desired salt content is obtained.

Grading.—Pickles are sorted in order to meet the varied demands of the consumer. They may be graded for shape and size. Straight pickles constitute the highest grades; the nubs and crooked ones, a lower grade, while broken and poorly shaped pickles may be used in the production of sweet relish or chowchow.

Pickles 2 to 3 in. in length are classified as small pickles and are used for bottling and sweet pickles. Medium-sized pickles, those 3 to 4 in. long, are used in making dill or sweet pickles, while large pickles, 4 in. or more in length, are used principally for sour pickles.

Preparation of Pickles.—Although there is a large number of pickle products, only a few of them will be discussed here. The interested reader is referred for further details to the publications listed at the end of the chapter or to works on Food Technology.

Salt pickles, "immature cucumbers preserved in a solution of common salt, with or without spices,"[2] may be converted into sour pickles, sweet pickles, mixed pickles, or other pickle products.

Sour pickles are made from salt pickles that have been processed, drained, and covered with vinegar. A 45- to 50-grain vinegar, distilled vinegar being preferred usually, is added to cover the pickles. When very sour pickles are desired, it is customary to add a 10- to 15-grain vinegar first and after a few days to replace it with the desired strength of vinegar, which may be as high as 60 to 75 grains.

Genuine dill pickles possess a fine flavor that is the result of aging and of biochemical changes brought about by microorganisms (principally bacteria) during fermentation, as well as the use of dill leaves (*Anethum graveolens*) to give the characteristic flavor.

[1] Blum, H. B., and F. W. Fabian, *Fruit Products Jour.*, **22** (No. 11): 326-329, 347 (1943).

[2] *U. S. Dept. Agr. Service and Regulatory Announcements, Food Drug Admin.*, No. 2, Rev. 5, November, 1936.

The method for preparing dill pickles differs from that of preparing salt pickles in two important respects: a weaker brine is used, and spices are added to the cucumbers. The spices do not interfere with the normal fermentation, but the salt concentration of the brine is low enough to permit the growth of undesirable microorganisms, hence special precautions should be observed in the manufacture of dill pickles.

Dill pickles are usually prepared in barrels that have been carefully cleaned and sometimes paraffined. Part of the dill (green, dry, or brined) and the other spices are placed at the bottom of the barrel. Cucumbers are added and the rest of the spice distributed evenly at the middle and top of the barrel. The barrel may or may not be headed, brine is added, and the barrel incubated at a temperature of around 70°F. (21.1°C.).

The addition of sugar, 2 lb. per barrel, favors the early development of bacteria. Sufficient vinegar or acetic acid to bring the pH to 4.6 is desirable, while the addition of a starter is advocated.

One formula for dill pickles calls for the use of 1 lb. of the following mixture in a 45-gal. barrel: 4 lb. of whole allspice, 2 lb. of coriander or mustard seed, 2 lb. of black pepper, and 1 lb. of bay leaves. From 6 to 8 lb. of green or brined dill, or 3 to 4 lb. of dried dill, are added to the same barrel, while a 7 to 8 per cent brine is commonly used.

In the dill-pickle fermentation, Gram-positive cocci predominate[1] at the beginning. Short rods, the majority of which are Gram-positive, replace the cocci. Toward the end of the fermentation, long rods predominate, the majority of these being Gram-positive. In approximately 8 to 10 days after the start of the fermentation the strong-acid-producing bacteria are at a maximum.

Imitation dill pickles may be prepared from salt pickles that have been processed, soured, drained, and placed in tight containers with dill, other spices, vinegar, and brine. The acid of imitation dill pickles is principally acetic acid, while in genuine dill pickles the acid is mainly lactic acid.

Sweet pickles are "pickled cucumbers or other vegetables sweetened with sugar and/or dextrose."[2] They may be prepared from salt pickles that have been soured and drained. A sirup is made by dissolving 4 to 10 lb. of sugar in 1 gal. of vinegar. The pickles are placed in a container, and one-half their weight of sirup is poured over them, and spices are usually added. At the end of 2 to 4 months, the sugar is largely inverted and the pickles have become darker green in color.

[1] FABIAN, F. W., and L. J. WICKERHAM, Mich. State Coll., Agr. Expt. Sta., Tech. Bull. 146, July, 1935.

[2] U.S. Dept. of Agriculture, Service and Regulatory Announcements, Food Drug Admin., No. 2, Rev. 5, November, 1936.

Wadsworth and Fabian[1] have discussed the use of lactic acid in combination with acetic acid in the finishing of sweet pickles, processed dill pickles, and pickle products. Ratios of lactic acid to acetic acid of 1:4 (0.4 per cent of lactic acid, as acetic acid, to 1.6 per cent acetic acid), or 1:9 produced the best results.

The influence of sugar and acid on flavors in pickles was determined by a panel of about 200 people in a study made by Switzer and Fabian.[2] There was a slight preference for a combination of 75 per cent sucrose and 25 per cent dextrose, rather than for sucrose alone, in a 20°Bé. sirup of 20-grain acidity. The tasters in general were more sensitive to slight increases in the acidity than they were to slight decreases in the sweetness.

Mixed pickles consist of two or more vegetables in the same container —cucumbers, cauliflower, green peppers, onions, beans, or green tomatoes being common vegetables used in the manufacture of mixed pickles.

Kosher dills are dill pickles containing garlic as an important spice.

Bread-and-butter Pickles.—Cucumbers of 1,200 to 1,800 count[3] (and usually onions) are soaked in potable water, washed in a rotary washer, and sliced. The slices are placed in a tank that has been thoroughly cleaned, covered with a brine of 25 to 30°salinometer, and permitted to stand overnight (about 12 hr). The slices are placed in 12 5- or 25-oz or other types of glass jars, each containing mustard and celery seed. A hot (160 to 170°F.) spiced sirup is poured over the slices and the glass jars are sealed. The pickles are pasteurized at 165°F for 15 min or at 160°F. for 20 min. and then cooled promptly.

Pasteurization is essential since acid-formers and yeasts develop during the brine treatment[3] and may survive the application of the hot liquor thus causing spoilage of the pickles. The sporeformers, which originate from the cucumbers or from the adherent soil, survive pasteurization, but show little or no increase during storage and are not therefore a source of spoilage. Moldy cucumbers, regardless of the degree, should not be used for making fresh cucumber pickle, since the mold produces off-flavors.

Pasteurization of Pickles.—Pickles are pasteurized to prevent undesirable changes in texture and flavor and spoilage from taking place. However, it is not essential to pasteurize all types of pickles. Salt stock, for example, may keep satisfactorily for several years if the brine concentration is maintained at a sufficiently high level, usually at 60 to 70° salinometer. Sour pickles keep on account of their vinegar content; and sweet pickles, because of their combined vinegar and sugar

[1] WADSWORTH, C. K., and F. W. FABIAN, *Food Ind.*, **11:** 252, 324 (1939).
[2] SWITZER, R. G., and F. W. FABIAN, *Food Ind.*, **12** (No. 11) 38 (1910).
[3] ETCHELLS, J. L., and H. B. OHMER, *Fruit Products Jour.*, **20** (No. 11) 334 337, 357 (1941).

TABLE 64.—GUIDE TO THE BACTERIOLOGICAL EXAMINATION OF CERTAIN BRINED, SALTED, AND PICKLED VEGETABLES AND VEGETABLE PRODUCTS[1]

Microbial group involved	Culture medium used (and abbreviation)	Classes of fermenting products in which microbial group is likely to be present[2]	Remarks concerning microbial groups
Coliform bacteria	Brilliant green lactose bile agar (BG) or violet red bile agar	IA: Fermenting salt stock vegetables and genuine dills; IIA and B: Brined and salted vegetables for non-pickle use	Gaseous fermentation. Group salt-tolerant but not acid tolerant. Most likely absent from finished pickles due to acid content; same is true for brines when appreciable acid is present
Acid-forming bacteria	Nutritive caseinate agar (NC)	IA: Fermenting salt stock vegetables and genuine dills; IB: Finished pickle products; IC: Pasteurized pickle products	Acid fermentation. Salt-tolerant up to 15 per cent; not likely to be found in brined and salted vegetables above this concentration (IIA and B)
Salt-tolerant cocci	Nutritive caseinate agar	IIA and B: Brined and salted vegetables for non-pickle use, also, other high-salt vegetables without appreciable acidity	No outstanding characteristics of fermentation reported. Group salt-tolerant but sensitive to acid. Can grow at refrigerator temperature (17°C) at approximately 10 per cent salt
Yeasts, mycoderma[4] and molds	Acidified dextrose agar (AD)	All classes of products (IA, B, and C, and IIA and B) for yeasts. Molds and mycoderma on liquid surface of products exposed to air and sheltered from sunlight	Yeasts: gaseous fermentation, acid- and salt-tolerant. Molds and mycoderma, acid- and salt-tolerant, both groups utilize acid of products and require free oxygen for growth
Obligate halophiles	Liver broth plus salt (LBS)	IIA and B: Brined and salted vegetables for non-pickle use. Also, in other vegetable brines at high-salt concentration	Gaseous fermentation. Group requires about 15 per cent salt in culture medium and reduced oxygen tension. Sensitive to acid. General fermentation behavior not well known
Butyric acid group	Liver broth plus particles (without salt) (LB)	Uncommon in brined and salted vegetables, examination should be made if malodorous fermentation is detected	Malodorous, gaseous fermentation. Not particularly acid- or salt-tolerant. Active fermentations rare in properly brined or salted vegetables

[1] ETCHELLS, J. L., L. D. JONES, *Am. Jour. Pub. Health*, **36** (No. 10): 1112 (1946).

[2] Also used for total plate count.

[3] Refer to outline for more detailed classification of products listed under IA, B, and C, and IIA and B

[4] Refers to film-forming yeasts in general.

microscopic examination of the samples and for the preparation and use of differential media.

The pickles and pickle products listed in the following classification, reproduced from the article by Etchells and Jones,[1] are considered in the guide presented in Table 94

1. Cucumber Pickles and Similar Pickle Products
   A. Salt Stock for Cured Pickle Products
      1 Cucumbers (and onions, peppers, tomatoes, cauliflower, melon rinds, etc)
      2. Genuine dill pickles (from cucumbers or tomatoes)
   B. Finished Pickle Products from Brine-cured Stock
      1. Sweets
      2. Sours
      3 Mixed
      4. Relishes
      5 Artificial or processed dills
   C. Types of Pasteurized Pickles (Not Brine-cured)
      1 Dills (sliced or whole)
      2 Sweets (sliced or whole)
      3 Relishes (mixed vegetable)
      4 Vegetables other than cucumbers (onions, peppers, tomatoes, etc)
II Brined and Salted Vegetables for Non-pickle Use
   A Brined
      1 Okra (whole)
      2 Celery (whole)
      3 Sweet pepper hulls
   B Dry-salted
      1 Corn
      2 Lima beans
      3. Peas
      4 Snap beans
      5. Okra (cut)
      6 Celery (cut)

The salinometer (or salometer) is an instrument that is very useful in measuring the salt concentration of a brine. The scale on the salinometer is divided into 100 degrees, the zero graduation indicating the reading of the instrument when placed in pure water at 60°F. and the 100° graduation indicating the reading obtained in a saturated solution of sodium chloride at 60°F. (26 5 per cent salt) Each degree represents slightly more than one-quarter of 1 per cent (0 265 per cent) of salt. Table 95 gives a comparison of salt percentages and the corresponding salinometer readings

PICKLED GREEN OLIVES

Briefly, the production of pickled green olives consists of selecting suitable varieties, harvesting, grading, treating with lye, washing with

   [1] *Ibid*

water, brining, and packaging. **In order to obtain a high-grade product,** it is necessary to use considerable care in each of the various operations

Varieties.—Cruess[1] lists five varieties of olives that are suitable for green pickling. These include the Sevillano, Barouni, Manzanillo, Ascolano, and Mission olives, the descending order of their suitability being given.

Harvesting.—The olives should be picked when full sized but free of any pink or red color. Green to partially yellow olives are preferred Olives that are straw-yellow to pink in color do not produce the best pickled products.

Grading.—The overripe, bruised, or blemished olives are removed and, if the olives will not be bruised in the process, they are graded, otherwise grading is deferred until after the fermentation.

Lye Treatment.—Olives are treated with a dilute lye (sodium or potassium hydroxide) to remove a portion but not all of the bitter principle, for some bitterness is desirable for flavor.

The concentration of lye used depends upon the variety of olive being treated. A 1.00 per cent lye, or one slightly less concentrated, is advocated by Cruess for Sevillano and Ascolano olives, but a 1.7 to 2 per cent lye is advocated for Manzanillo, Barouni, and Mission olives because they contain more of the bitter principle. The use of too concentrated lye or prolonged treatments will remove all the bitter principle and impair the flavor, color, and texture.

The time required for treating the olives with lye is dependent on the temperature of the process, the concentration of the alkali, the size of the olives, and depth of penetration desired. The temperature of the lye is generally 70 to 75°F. (21.1 to 23.9°C.). Lye is permitted to penetrate one-half way to the pit in Sevillano and Ascolano olives and two-thirds of the way in Manzanillo, Barouni, and Mission olives The process usually requires 4 to 6.75 hr., during which time the olives should be stirred occasionally.

The progress of lye penetration is followed by removing olives from time to time, slicing them, placing a few drops of phenolphthalein on the cut surface and observing the pink to red color that indicates the depth to which the lye has penetrated.

Washing.—The lye-treated olives are washed to remove the alkali. The olives are covered with clean water which is allowed to stand for perhaps 2 or 3 min. and then drained off. This process is repeated for perhaps three times, and thereafter the water is changed about once every 3 hr. Ordinarily about 30 hr. may be required for washing, but the actual time will depend on the frequency of changing the water, the size of the

[1] Cruess, W. V, *Calif. Agr. Sta., Bull.* 498, October, 1930.

Table 95.—Salt Percentages and Corresponding Salinometer Readings

Salt in solution, per cent	Salinometer reading, degrees	Salt in solution, per cent	Salinometer reading, degrees
0	0	10 07	38
0 265	1	10 6	40
0 53	2	11 13	42
0 795	3	11 66	44
1 06	4	12 19	46
1 325	5	12 72	48
1 59	6	13 25	50
1 855	7	13 78	52
2 12	8	14 31	54
2 385	9	14 84	56
2 65	10	15 37	58
2 915	11	15 9	60
3 18	12	16 43	62
3 445	13	16 96	64
3 71	14	17 49	66
3 975	15	18 02	68
4 24	16	18 55	70
4 505	17	19 08	72
4 77	18	19 61	74
5 035	19	20 14	76
5 3	20	20 67	78
5 565	21	21 2	80
5 83	22	21 73	82
6 095	23	22 26	84
6 36	24	22 79	86
6 625	25	23 32	88
6 89	26	23 85	90
7 155	27	24 38	92
7 42	28	24 91	94
7 65	30	25 44	96
8 48	32	25 97	98
9 01	34	26 5	100
9 54	36		

olives, the depth of penetration of the lye in the olives, and the concentration of the lye

One does not expect to remove all the lye from all the olives during washing When 75 to 80 per cent of the olives show no pink or red color with phenolphthalein when this indicator is applied to the cut surface, a satisfactory removal of lye is believed to have taken place

Fermentation—For brining and fermentation the olives should be placed in oak barrels A 44° salinometer (11 66 per cent salt) brine is added at once to Manzanillo Barouni, or Mission olives Sevillano

and Ascolano olives will shrivel if a concentrated brine is used. Hence Cruess advocates adding a 20° brine to the olives and increasing the concentration by 5° at 2-day intervals until the concentration remains fairly constant at 28 to 30° salinometer. A final concentration of less than 27° salinometer is considered dangerous, for gas tends to form in the olives, likewise spoilage, including softening, increases. The use of a brine more concentrated than 32° produces olives that are too salty in flavor.

The addition of 3 lb. of dextrose, dissolved in the brine, to a 50-gal. barrel increases the acidity of Manzanillo and Mission olives. Sugar should not be added to the other varieties during the fall of the year.

Starters may be added along with the brine, for during the lye treatment a large part of the lactic acid and other bacteria are destroyed. Cruess,[1] experimenting with a number of lactobacilli grown in filtered and sterilized tomato juice, advocates the addition of starters containing pure cultures to green olives. He found *Lactobacillus pentosus* (probably a synonym of *L. plantarum*)[2] to be most desirable, for olives inoculated with this organism possessed a better flavor and higher acidity than uninoculated controls. *L. pentoaceticus* (probably a synonym of *L. brevis*)[2] likewise gave fairly good results. The brine from olives that are normal in flavor and odor may be used as a starter. Such brine should contain the fewest possible number of film yeast or mold spores, otherwise its use may cause much more harm than good.

Acidification of the brine with a small amount of vinegar or lactic acid has proved of value in improving the quality of the pickled olives

Care during Fermentation.—Barrels containing the olives are commonly placed in the sun to ferment, although they may be incubated at a temperature of 70 to 80°F. (21.1 to 26.7°C.). Brine or salt is added several times a week for the first 3 or 4 weeks and thereafter about once a week to maintain a concentration of 28° salinometer and to keep the barrel full. Unless the barrel is kept filled and sealed, film yeast and molds will develop and utilize the acid produced during fermentation. Since the flavor and keeping qualities of the olives depend in large part on the acidity of the brine, the necessity for preventing the growth of the yeast film and molds is evident.

Sugar may be added to the brine if the acidity fails to increase to 0.9 g. or greater per 100 cc.

Packaging.—The olives are packaged in glass jars after the flesh has lost its "chalky-white" color, a characteristic flavor has developed, and the acidity is equivalent to 0.9 g. per 100 cc., or greater. After the jars

[1] Cruess, W. V., *Fruit Products Jour.*, **17**: 12 (1937).
[2] "Bergey's Manual of Determinative Bacteriology," 6th ed , The Williams & Wilkins Company, Baltimore, 1948.

are filled with olives, water is added to rinse away any sediments. The jars are, after draining, filled then with a 30° salinometer brine, and lactic acid may be added if the original brine contained less than 0 5 g. of acid per 100 cc. Sealing is effected, with or without the use of vacuum.

References on Pickles and Pickling

BEATTIE, J. H.: Growing Cucumbers for Pickling, *U.S. Dept. Agr*, *Farmers' Bull* 1620, 1930.

BINSTED, R H.. "Pickle and Sauce Making," R H. Binsted, London, 1939

BLUM, H B, and F. W FABIAN. The Influence of Salting Upon Vitamins A and C in Vegetables, *Fruit Products Jour*, 22 (No 9) 273–275, 283 (1913).

—— and ——; Spice Oils and Their Components for Controlling Microbial Surface Growth, *Fruit Prod Jour*, 22 (No 11): 326–329, 317 (1913).

BROWN, C W. Notes on Brine Pickle Fermentation, *Jour. Bact*, 1: 104 (1016).

CAMPBELL, C. H : Changes That Take Place during the Salting of Pickles, *Canner*, 50: 45 (1920)

—— "Canning, Preserving, Pickling," The Canning Age Publishing Co , 1937.

CRUESS, W V Pickling Green Olives, *Calif. Agr. Expt. Sta , Bull.* 498, October, 1930

—— Use of Starters for Green Olive Fermentations, *Fruit Products Jour.*, 17: 12 (1037)

—— "Commercial Fruit and Vegetable Products," 3d ed , McGraw-Hill Book Company, Inc , New York, 1948

—— Olive Products, *Ind Eng Chem*, 33: 300–303 (1941).

ETCHELLS, J L. Rate of Heat Penetration During the Pasteurization of Cucumber Pickle, *Fruit Products Jour*, 18 (No 3). 68–70 (1938).

—— The Incidence of Yeast in Cucumber Fermentations, *Food Res*, 6 (No 1): 95–104 (1941)

—— A New Type of Gaseous Fermentation Occurring during the Salting of Cucumbers, *University Microfilms, Publ.* 282, 1–153, Ann Arbor, Mich (1941).

—— , F W FABIAN, and I D JONES: The *Aerobacter* Fermentation of Cucumbers During Salting, *Mich State Coll Agri Expt Sta , Tech Bull* 200, June, 1915

—— and H E GONZALEZ. Methods of Examination of Fresh Cucumber Pickle, *Fruit Products Jour* , 19 (No. 11) 331–335 (1940)

—— and I D JONES. An Occurrence of Bloaters During the Finishing of Sweet Pickles, *Fruit Products Jour* , 20 (No 12) 370, 381 (1941).

—— and ——: Pasteurization of Pickle Products, *Fruit Products Jour* , 21 (No 11) 330–332 (1942)

—— and ——: Mortality of Microorganisms during Pasteurization of Cucumber Pickle, *Food Research*, 6 (No 1) 33–51 (1941).

—— and ——: Bacteriological Changes in Cucumber Fermentation, *Food Inds* , 15 (No 2) 54–56 (1943)

—— and ——: Commercial Brine Preservation of Vegetables, *Fruit Products Jour* , 22 (Nos 8 212 216 251, 253 (1943)

—— and ——: The Importance of Care in the Pasteurization of Pickle Products, *Canner*, 98 (No 4) 28, 61 (January, 1944)

—— and ——: Preservation of Vegetables by Salting or Brining, *Farmers' Bull* 1932 (revised June, 1944) (This bulletin contains a list of other publications on the subjects of salting and brining)

———— and ————: Procedure for Pasteurizing Pickle Products, *Glass Packer*, July, 1944.

———— and ————: Procedure for Bacteriological Examination of Brined, Salty, and Pickled Vegetables and Vegetable Products, *Am. Jour. Pub. Health*, **36** (No. 10): 1112–1123 (1946).

———— and H B OHMER: A Bacteriological Study of the Manufacture of Fresh Cucumber Pickle, *Fruit Products Jour.*, **20** (No. 11): 334–337, 357 (1941)

———— and M K VELDHUIS: Growth of Mycoderma Scum under Oil, *Fruit Products Jour.*, **18** (No. 9): 265–267, 280 (1939).

————, ———— and O. VEERHOFF: Pasteurization of Genuine Dill Pickles, *Fruit Products Jour.*, **20** (No. 10): 304 (1941).

FABIAN, F W : Some Important Considerations in Pickle Manufacture, *Fruit Products Jour.*, **9** : 376 (1930).

————: Pickle and Kraut Packers Conference, *Fruit Products Jour.*, **16**: 209, 214, 217, 240 (1937); **17**: 238, 270 (1938)

————: A New Method for the Prevention of Scum-forming Organisms, *Fruit Products Jour* , **19** : 214 (1940).

————: Pickles–Tests Show Chemical Action of Spice Oils in Pickles, *Western Canner & Packer*, November, 1942

————: Spicing Pickles, *Western Canner & Packer*, December, 1942

————: Wartime Vegetable Salting, *Food Packer*, **16** (No. 5); (1944)

———— and H B BLUM: Preserving Vegetables by Salting, *Fruit Products Jour.*, **22** (No 8): 228–236 (1943)

———— and C. S BRYAN: An Attempt to Prevent the Formation of Mycoderma Scums on Pickle Brine by the Use of a Mercury Vapor Lamp, *Fruit Products Jour* , **11** : 134 (1932)

————, ———— and J L ETCHELLS: Experimental Work on Cucumber Fermentation, I.-V., *Mich State Coll Agr Expt Sta* , *Tech Bull* 126, November, 1932

———— and V HARRIS: Imitation Clove Oil Effective in Spicing Pickles, *Food Inds* , **17** : 638–640 (1945)

———— and E. A. JOHNSON: Experimental Work on Cucumber Fermentation, *Mich State Coll* , *Agr Expt Sta* , *Tech Bull* 157, February, 1938.

————, C F KREHL, and N W LITTLE: The Role of Spices in Pickled-food Spoilage, *Food Research*, **4** (No. 3): 269–286 (1939).

———— and A. L. NIEBHUIS: Experimental Work on Cucumber Fermentation, VI, VII, *Mich. State Coll* , *Agr Expt Sta.*, *Tech. Bull.* 140, August, 1934.

———— and R G. SWITZER: Experimental Work on Processing and Finishing of Sweet Pickles III. Use of Dextrose and Sucrose in Manufacture of Sweet Pickles, *Proc. Inst. Food Technol* , 329–382 (1940).

———— and ————: Classification of Pickles, *Fruit Products Jour* , **20** (No 5): 136–140 (1941)

———— and M C VAN WORMER: Factors Influencing the Flavoring of Pickles with Whole Spices and Essential Oils, *Food Research*, **8**: 95–104 (1943).

———— and C. K WADSWORTH: The Utilization of Green Tomatoes, *Fruit Products Jour* , **23** (No 12): 361–362 (1944).

———— and ————————————— Salting Beets, Carrots, Corn, Green Beans and Spinach, Diamond Crystal Salt Co , St Clair, Mich.

———— and L J WICKERHAM: Experimental Work on Cucumber Fermentation, VIII, *Mich. State Coll.*, *Agr. Expt Sta* , *Tech. Bull.* 146, July, 1935

JONES, I D : Salting of Cucumbers, *Ind Eng Chem* , **32**: 858–861 (1940)

——— and J. L. Etchells: Physical and Chemical Changes in Cucumber Fermentation, *Food Inds*, 18 (No. 1): 62–64 (1913).

——— and ———: Food Value of Brined Vegetables, *Research & Farming* (October, 1945). (Refer also to *Am. Jour. Pub. Health*, 34: 711–718 (1944))

———, ———, M. K. Veldhuis, and O. Veerhoff: Pasteurization of Genuine Dill Pickles, *Fruit Products Jour.*, 20 (No. 10): 301–305, 316, 325 (1941)

———, M K. Veldhuis, J L. Etchells, and O. Veerhoff: Chemical and Bacteriological Changes in Dill-Pickle Brines during Fermentation, *Food Research*, 5 (No 5): 533–547 (1940)

Joslyn, M A : Some Observations on the Softening of Dill Pickles, *Fruit Prod. Jour*, 8 : 19 (April), 8 : 16 (May) (1929)

——— and W V. Cruess. Home and Farm Preparation of Pickles, *Calif. Agr. Ext. Ser*, *Circ.* 37, 1929.

Le Fevre, E : Bacteriological Study of Pickle Softening, *Canner*, 48: 205 (1919)

——— Pickle Processing Investigations. *Canner*, 60: 230 (1925)

———. Bacteriology of Sauerkraut and Pickles, *Chem. Age (N Y)*, 30: 24 (1922)

———: Elimination of Diseased or Dirty Cucumbers from Pickle Curing Tanks, *Canner*, 62 (Part II): 171 (1926).

——— Making Fermented Pickles, *U S. Dept. Agr*, *Farmers' Bull* 1438, April, 1927.

Leslie, B E , and W V Cruess: The Effect of Acidity on the Softening of Dill Pickles, *Fruit Products Jour* , 7 : 12 (1928)

Littlejohn, R M , and R T Willkie: Pickles, Olives and Sauerkraut, *The Quartermaster Corps Subsistence School Bull* 44, Series X, 1928

McKinnon, J S , and C C Morris Jelly, Preserves, Jam, and Pickles, *N C. Agr Ext Ser*, *Ext Circ* 113, May, 1937

Switzer, R G , and F W Fabian 200 Tell What They Prefer in Pickle Sweeteners, *Food Inds* , 12 (No 11) 38–40 (1910)

Rahn, O salt for Curing Cucumbers, *Canner*, p 40 (September, 1929)

Tanner, F W , and I O Eagle Research in Pickle Fermentation, *Canning Age*, 7: 651, 713, 783 (1926)

Vaughn, R H , H C Douglas, and J R Gilliland: Production of Spanish-type Green Olives, *Univ Calif , Coll of Agr* , *Bull* 678, April, 1943

Veldhuis, M K and J L Etchells Gaseous Products of Cucumber Pickle Fermentations, *Food Research*, 4 (No 6) 621–630 (1939)

———, ——— , and I D Jones, and O Veerhoff Influence of Sugar Addition to Brines in Pickle Fermentation, *Food Inds* , 13 (No 10) 51–56, 13 (No 11) 48–50 (1941)

Wadsworth, C R , and F W Fabian How to Use Lactic Acid in Finishing Pickles and Pickle Products, *Food Inds* , 11: 252, 321 (1939)

——— and ——— Salting Early June and Alaskan Peas *Fruit Prod Jour* , 23 (No 10): 298, 301, 316 (1944)

Wentzel, C W , and F W Fabian Experimental Work on Cucumber Fermentation XIII Influence of Garlic on the Softening of Genuine Kosher Dill Pickles, *Mich State Coll , Agr Expt Sta , Tech Bull* 191, February, 1943

West, N S , J R Gilliland, and R W Vaughn Characteristics of Coliform Bacteria from Olives, *Jour Bact* 41: 341–352 (1941)

Wonnley, W G Forty Years of Pickle Salting, *Food Inds* 11 486, 551 (1939)

CHAPTER XIX

SOME LACTIC ACID BACTERIA AND SOME FERMENTED MILK PRODUCTS

Lactobacillus acidophilus.—*L. acidophilus* has become important in the therapy of various disorders of the gastrointestinal tract, such as constipation, on account of the fact that its successful implantation in the intestines may often relieve the symptoms associated with some of these disorders. When the flora of the intestines is largely putrefactive in nature certain symptoms, such as headaches, may appear. The predominance of *L. acidophilus* in the intestines, along with lactic acid, which it produces, reduces or inhibits the development of the putrefactive types.

It must be emphasized however that, whereas the implantation of *L. acidophilus* has produced very definite relief in certain cases, it is not a cure-all. Successful therapy depends upon careful attention to certain essential details, which will be discussed later.

Description of Organism.—*L. acidophilus* is a member of the important genus, *Lactobacillus*. The bacteria occur as rather slender rods, about 0.6 to 0.9 by 1.5 to 6 μ,[1] singly, in pairs, or in short chains. It is Gram-positive in young cultures but may show Gram-negative forms in older cultures. It is nonsporeforming, nonmotile, microaerophilic, an inhabitant of the intestines of animals, especially herbivores, and has an optimum temperature of 37°C. It produces acid from galactose, glucose, lactose, levulose, maltose, mannose, and sucrose. Some strains ferment raffinose and trehalose, and occasionally dextrin.

There are two main types of the intestinal strain of *L. acidophilus*, although intermediate varieties exist. One produces rough colonies and is referred to as the X or R type, while the other produces smooth colonies and is recognized as the Y or S type. Although R strains may be readily dissociated to S strains, the reverse procedure is very difficult or impossible to achieve.

The strain of *L. acidophilus* that produces rough colonies is of proved therapeutic value and occurs more commonly in the normal human intestines, hence this strain is used commercially in preparing cultures for therapeutic use.

[1] "Bergey's Manual of Determinative Bacteriology," 6th ed., The Williams & Wilkins Company, Baltimore, 1948.

Frequent isolation of the organism from the human intestinal tract favors an active culture that may be implanted readily under favorable conditions.

Conditions Necessary for Implantation.—In order to implant *L. acidophilus*, it is essential to select a strain that will develop in the human intestines. Likewise the organism should be able to multiply rapidly in favorable carbohydrate media and produce a fine coagulum in milk.

Implantation is favored by the ingestion of very large numbers of the organism. When acidophilus milk is used, the dosage recommended is about 1,000 cc. per day. Since the milk must contain at least 100 million viable bacteria per cubic centimeter at the expiration date, it is evident that enormous numbers are consumed.

A second very important factor in successful implantation is the administration of considerable amounts of lactose or dextrin at the time the culture is taken. The use of at least 100 g. of lactose or dextrin daily is advocated, especially if a culture or concentrate of *L. acidophilus* is used rather than milk. Milk, of course, contains lactose as a normal constituent.

L. acidophilus Preparations.—Acidophilus milk is perhaps the most common and preferable preparation. Broth cultures and concentrates of the organism in candy form or combined with yeast have appeared on the market, but are less effective.

Acidophilus Milk.—Acidophilus milk is prepared by inoculating sterilized, fresh whole milk, or partially skimmed milk, with a pure culture of a selected strain of *L. acidophilus*. The milk is sterilized at 120°C. for 15 min. or at lower temperatures for longer periods of time. It is important not to injure the flavor, composition, or appearance of the milk by overtreating it with heat. Pasteurization of milk is not satisfactory, for the types of bacteria that survive pasteurization develop rapidly at the incubation temperature of *L. acidophilus* (35 to 37°C.) and may predominate. The inoculated milk is incubated for 20 to 18 hr. at 35 to 37°C. until a characteristic product is obtained. Usually the fermentation is permitted to proceed until a definite acidity is produced. Some manufacturers consider the fermentation complete when 8 to 10 cc. of N/10 sodium hydroxide solution are required to neutralize a 10-cc. portion of the milk when phenolphthalein is used as the indicator. The lactic acid present in a well-ripened milk is usually 0.65 to 0.75 per cent.

The final product should be slightly sour in flavor and should possess an odor resembling buttermilk. In order to improve the consistency, the product is usually homogenized. It is then bottled and stored at a

then bottled and stored at relatively low temperatures to prevent increases in acidity and undesirable changes in flavor or consistency.

Kefir is a fermented milk product that originated in the Caucasus Mountains. It is extensively used as a food by the natives of that region It is prepared by inoculating the milk of mares, goats, ewes, or cows with small convoluted masses of "seed," known as kefir grains. These grains resemble to some extent miniature cauliflowers and may be dried and preserved. Kefir grains usually contain *Lactobacillus casei*, *streptococci*, and a lactose-fermenting yeast, *Saccharomyces kefir*, besides other microorganisms. Since the fermentation is usually carried out in goatskin bags or closed glass bottles, the product becomes effervescent, owing to the evolution of gas Lactic acid and alcohol are two important fermentation products.

Kefir has also been known as *kefyr, kephir,* and *kephyr.*

A product closely related to the original kefir is made by adding sucrose (about 4 per cent) and a culture of yeast to buttermilk in a covered glass bottle. It is incubated at 70°F. (21.1°C.) until a desirable end point is reached and is then cooled to stop the growth of the organisms.

Directions for making Kefir fermented milks have been prepared by Burkey (1947), of the Bureau of Dairy Industry, U.S. Department of Agriculture.

Yoghurt is a fermented milk product used by Bulgarian tribes. It is primarily a soured milk but occasionally may contain some alcohol. *Lactobacillus bulgaricus* is the dominating organism found in yoghurt, although it is the product of a mixed flora of microorganisms.

Metchnikoff, who was amazed at the longevity of the tribes that used yoghurt regularly, studied this fermentation and publicized an account of it in his book, "The Prolongation of Life."

Yoghurt is likewise known as *yogurt, yoghourt, yahourth, yohouri,* or *yahourd.*

Kumiss is a fermented milk product that originated in a Russian tribe, known as Kumanes, living near the Kuma River. It is usually prepared from mare's or cow's milk and contains lactic acid and alcohol. The fermentation, brought about by a mixed flora, in which, according to Rogers,[1] lactic streptococci, lactobacilli of the *L. casei* type, and a lactose-fermenting yeast are the predominating organisms, is carried out in skin or leathern bags or in open vessels. If the fermentation proceeds in a closed container an effervescent product is obtained, owing to the retention of the gas evolved.

Kumiss is sometimes known as *kumyss, kumys,* or *koumiss.*

[1] Rogers, L. A (associates of), "Fundamentals of Dairy Science," 2d ed., Reinhold Publishing Corporation, New York, 1935

Leben is a fermented product made usually from cow's, goat's, or buffalo's milk. Bacteria and yeasts ferment the milk to produce acid and alcohol. Although this product has been native to Egypt for hundreds of years, where it is known as *leben* or *leben raib*, it is used in Syria, Sardinia, Algeria, and in other regions.

References on Lactobacillus Acidophilus

American Medical Association: "New and Nonofficial Remedies," Chicago, 1937.

Frost, W. D., and H. Hankinson. "Lactobacillus Acidophilus. An Annotated Bibliography to 1931," Davis-Greene Corporation, Wisconsin, 1931.

Kopeloff, L. M., J. L. Etchells, and N. Kopeloff: Bacteriological Changes in Acidophilus Milk at Room and Icebox Temperatures, *Jour. Bact.*, 28: 489-500 (1934).

—— and N. Kopeloff: Optical Activity of Lactic Acid Produced by *L. acidophilus* and *L. bulgaricus*, *Jour. Bact.*, 33: 331-331 (1937)

Kopeloff, N.: "Lactobacillus Acidophilus," The Williams & Wilkins Company, Baltimore, 1926.

——: Dissociation and Filtration of *Lactobacillus Acidophilus*, *Jour. Infectious Diseases*, 55: 368-379 (1934).

——: Acidophilus Milk Therapy, *Psychiat. Quart.*, 9: 20 (1935).

Longsworth, L. G., and D. A. MacInnes: Bacterial Growth with Automatic pH Control, *Jour. Bact.*, 29: 595 (1935)

—— and ——: Bacterial Growth at Constant pH, *Jour. Bact.*, 31: 287 (1936).

Rettger, L. F., M. N. Levy, L. Weinstein, and J. E. Weiss. "Lactobacillus Acidophilus and Its Therapeutic Application," Yale University Press, New Haven, 1935.

References on Fermented Milks

Berry, A. D.: "Practical Manufacture of Cultured Milks and Kindred Products," The Olsen Pub. Co., Milwaukee, 1938

Berry, L. A.: Cultured Buttermilk, BDIM-Inf-14, *Bur. Dairy Industry, U.S. Dept. Agr.*, August, 1941

——: Bulgarian and Acidophilus Cultured Milks, BDIM-Inf-16, *Bur. Dairy Industry, U.S. Dept. Agr.*, September, 1941

——: Directions for Making Kefir Fermented Milks, BDIM-Inf-58, *Bur. Dairy Industry, U.S. Dept. Agr.*, 1947.

——: How to Make Yoghurt, BDIM-Inf-2, *Bur. Dairy Industry, U.S. Dept. Agr.*, November, 1943.

Eckles, C. H., W. B. Combs, and H. Macy: "Milk and Milk Products," McGraw-Hill Book Company, Inc., New York, 1936

Hammer, B. W.: "Dairy Bacteriology," 3d ed., John Wiley & Sons, Inc., New York, 1948

Marshall, C. E.: "Microbiology," P. Blakiston's Son & Company, Philadelphia, 1921

Rogers, L. A. (associates of): "Fundamentals of Dairy Science," 2d ed., Reinhold Publishing Corporation, New York, 1935

Winton, A. L., and K. B. Winton: "The Structure and Composition of Foods," Vol. III, "Animal Products," John Wiley & Sons, Inc., New York, 1937

CHAPTER XX

CHEESE

Cheese was prepared in Asia and in Europe several hundred years, at least, before the birth of Christ. A study of the origin of some of the many different types of cheeses lies outside the scope of this text, but the interested reader will derive considerable enjoyment and add to his knowledge by perusing the history of cheese making.

Definition.—Cheese, according to the definition and standards of the Food and Drug Administration of the U.S. Department of Agriculture,[1] is:

. . . the product made from the separated curd obtained by coagulating the casein of milk, skimmed milk, or milk enriched with cream. The coagulation is accomplished by means of rennet or other suitable enzyme, lactic fermentation, or by a combination of the two. The curd may be modified by heat, pressure, ripening ferments, special molds, or suitable seasoning.

The name "cheese" unqualified means Cheddar cheese (American cheese, American cheddar cheese).

The Milk.—Milk from ewes (sheep), goats, cows, mares, and other animals has been used for the making of cheeses. Each of these milks contains fats, protein, milk sugar (lactose), mineral salts, and water. Fat is present as an emulsion, but the sugar, minerals, and some of the proteins are soluble in the water of milk. Casein (the principal protein) is combined with calcium and exists in a colloidal condition.

Formation of Curd.—If acid is produced in milk as a result of the fermentation of lactose by bacteria, the casein is freed from calcium and no longer appears as fine particles distributed throughout the medium, but instead goes out of solution and eventually accumulates in large lumps of curd or as a continuous mass.

Rennet, an enzyme, likewise has the ability to alter the colloidal condition of casein, causing coagulation or curdling. This enzyme, sometimes known as "rennin," is obtained commercially by extracting the fourth stomach of the calf where it is principally found. Commercial pepsin sometimes serves as a successful substitute for rennet.

[1] *Service and Regulatory Announcements, Food Drug Admin.*, No. 2, Rev. 5, Nov. 5, 1936.

Whey.—Whey is the watery portion of milk separated from the curd during cheese making. The principal constituents of whey are lactose, soluble ash, and lactalbumin. A small amount of fat is a normal component of whey also.

Treatment of Curd.—The curd formed in milk through the action of rennet, lactic acid, or a combination of the two, may be treated in a variety of manners. After it has settled into a fairly compact jelly-like mass, it is usually cut into small pieces by special knives to facilitate drainage or removal of occluded whey. It may be drained with or without eventual pressure, depending on the type of cheese being manufactured. The curds of the soft or semisoft cheeses are pressed just sufficiently to remove the excess of whey, while those of the hard cheeses may be heated and pressed in a form with weights until the curd forms a firm mat. The curd is treated with varying quantities of salt by various methods. Finally the treated curd is molded into the desired shape and is ready for ripening or curing.

Ripening.—During this process, the flavor, composition, and physical properties of the cheese undergo change due to the action of characteristic molds or bacteria and their enzymes. The nature of the changes depends on the method used in treating the curd, the method and quantity of salting, the microorganisms present or added, and the control of temperature and relative humidity in the curing rooms, chambers, or caves.

Classification.—Cheeses may be classified in several manners: on the basis of hardness, as hard, semihard, and soft cheeses; on the basis of the principal ripening agents, as mold or bacterial cheeses; on the basis of the kind of milk from which manufactured, as cow, ewe, goat, or other milk, on the basis of the country of origin, as Italian, French, American, and the like; on the basis of the method of coagulation, as rennet or acid curd cheese, on the basis of added substances, such as sage; or on the basis of the fat content of the milk, as full cream, whole milk, or skim-milk cheeses.

In Table 96 cheeses are classified as hard, semihard, and soft rennet cheeses, and as lactic cheeses. Sometimes Roquefort and Stilton cheeses are classified as hard cheeses and Brick, Brie, Camembert, Limburger, and Münster as soft cheeses, rather than as semihard cheeses. Blue cheese, Brie, Camembert, Gorgonzola, Roquefort, and Stilton are characterized as mold cheeses.

Analyses of Some Varieties of Cheese.—Table 97 contains the average analyses of some varieties of cheese.

Cheddar Cheese.—Cheddar cheese, a hard cheese prepared from whole milk by the action of rennet, originated in Cheddar, England. It is the

TABLE 96.—A CLASSIFICATION OF CHEESES

Name of Cheese	Place of Origin	Name of Cheese	Place of Origin
Hard Rennet Cheeses		Semihard Rennet Cheeses	
Cheddar . .	England	Limburger	Belgium
Cheshire (Cheddar type) .	Chester, England	Stilton (mold)	Stilton, England
		Brie (mold)	France
Cacioeavallo .	Southern Italy	Blue (mold)	France, Denmark, etc.
Gorgonzola (mold)	Italy (near Gorgonzola)		
		Camembert (mold) . . .	France
Parmesan	Italy	Roquefort (mold) . . .	France
Pecorino .	Italy	Münster	Near Münster, Germany
Provolone .	Italy		
Reggiano	Italy	Brick	United States
Romano . .	Italy	Soft Rennet Cheeses	
Edam	Edam, Holland		
Gjedost	Norway	Port du Salut	Canada
Emmenthaler	Switzerland	Neufchâtel	France
Gruyère (Swiss)	Gruyère, Switzerland	Soft Lactic Cheeses	
Pineapple .	United States	Cottage	
		Cream	United States

leading cheese manufactured in the United States and is widely made by the English

The milk used for the manufacture of cheddar cheese is analyzed, standardized, weighed, and heated to about 86°F. (30°C.), and a suitable starter of lactic acid bacteria is added. Vegetable coloring matter may be incorporated also at this time if it is to be used. The milk is thoroughly mixed, and when the acidity is correct (0.17 to 0.2 per cent) rennet (approximately 3 oz. for each 1,000 lb. of milk), diluted with water, is added. It requires only 20 to 40 min. to coagulate the casein. When coagulation is satisfactory, demonstrated by a clean breaking of the curd, leaving a clear whey, the curd is cut by special knives into cubes about ¼ in. square to permit the whey to escape more readily. The curd is slowly heated to about 100°F. (37.8°C.). Acidity and body are developed in the curd as it is cooked with gentle agitation to keep it from settling. The whey is drained off when the curd has assumed the desired texture.

The curd is now cut into large pieces, which are turned frequently and placed one on top of the other to force out the whey. This treatment is known as cheddaring.

Cheddared curd is then cut up and milled into small pieces by special machines. Salt, at the rate of approximately 3.5 lb. to each 1,000 lb. of

TABLE 97.—AVERAGE ANALYSES OF SOME VARIETIES OF CHEESE[1]

Variety	Authority	Water, per cent	Fat, per cent	Proteids, amides etc., per cent	Milk sugar, lactic acid, etc., per cent	Total ash, per cent
Brick	Bureau of Chemistry	42.47	30.66	21.05	....	2.95
Brie						
Imported	Bureau of Chemistry	52.53	22.44	20.94	...	4.81
American	Bureau of Chemistry	52.10	24.72	19.60	...	4.06
Cariocavallo	Bureau of Chemistry	34.95	21.99	34.33	..	6.96
Camembert imported	Bureau of Chemistry	47.88	26.32	22.21	...	4.11
Cheddar skim	Bureau of Chemistry	57.04	4.88	32.09	.	3.76
Cream-French Demi-sel	Bureau of Chemistry	42.74	39.88	14.49	.	1.88
Edam (American)	Bureau of Chemistry	36.07	22.65	30.89		6.19
Emmenthaler	Lindt	33.00	30.50	30.44		4.17
Gorgonzola	Museo	37.30	31.67	23.16	1.62	3.82
Gouda	Cribb	34.79	9.02	23.94		5.52
Gruyère	Ballard	29.09	25.19	33.01	4.82	3.96
Limburger						
American	Arnold	35.64	29.82	28.53		5.98
Imported	Bureau of Chemistry	54.79	19.61	21.27		5.17
Münster (American)	Bureau of Chemistry	40.60	31.00	22.20		4.63
Neufchâtel	Bureau of Chemistry	52.03	23.31	19.93	.	4.97
American	Bureau of Chemistry	59.22	18.17	21.30		2.43
Parmesan						
Formaggio	Bureau of Chemistry	16.95	22.71	49.39		7.59
Reggiano	Bureau of Chemistry	29.63	27.29	31.84		4.76
Leccino	Satori	29.80	30.31	31.51		8.24
Romano	Bureau of Chemistry	29.56	27.69	31.20		8.64
Pineapple	Johnson	24.07	38.12	29.35	2.43	3.61
Port du Salut	Bureau of Chemistry	50.10	23.17	21.18		2.91
Roquefort	Curne	38.69	32.31	21.39		6.11
Stilton	Bureau of Chemistry	33.57	31.19	28.95		3.90
Swiss						
American	Bureau of Chemistry	34.58	32.40	27.43		4.16
Imported	Bureau of Chemistry	33.91	30.61	29.22		4.16

[1] Data tabulated from U. S. Dept. Agr. Bul. 608 revised February 1912

curd, is added and carefully mixed with the curd. The product is cooled to 80°F. (26.7°C.), packed in hoops containing cheesecloth linings, and pressed for several hours to the desired shape.

During the ripening process, originally carried out in the Cheddar caves and which may require a few weeks or several months, the bacteria and enzymes present bring about characteristic changes in the cheese. Lactic acid bacteria, such as *Streptococcus lactis* and *Lactobacillus casei*, are especially important in the ripening or curing of the cheddar cheese. The bacteria increase enormously, often reaching several hundred millions in numbers per gram during the first few weeks, and then gradually die off until only a few million per gram remain at the end of 8 months.

It is necessary to prevent molds from growing on this cheese, for they cause a rapid deterioration of the product. This is done by use of salt on the surface

A yield of approximately 1 lb. of cheese is obtained from 10 lb of milk by this process.

Roquefort Cheese.—Roquefort cheese is a semihard, friable cheese characterized by a mottled or marbled appearance of the interior, due to the presence of *Penicillium roqueforti*, a blue-green mold. This cheese has been known for almost a thousand years, having originated in the south of France. It is made principally from the milk of ewes, bred particularly for their high milk-producing abilities. The sheep graze in the plateau region of the Cevennes. Their milk is made into cheese in small factories distributed over this relatively small section of Southern France. The cheese is transported to the vicinity of Roquefort for ripening

In making Roquefort cheese, the curd is drained in hoops but not pressed. It is inoculated at this time with a dried bread product rich in mold spores, the inoculum being prepared by growing *P. roqueforti* in the interior of bread in a moist cool place until the bread becomes a moldy mass containing a vast number of spores. The bread is dried carefully and ground to a powder, the latter being used as the inoculum.

During ripening, the temperature and relative humidity must be carefully controlled in order to obtain a f ie cheese The temperature of ripening must not be much higher than 48°F. (8.9°C.), and the relative humidity should be high. In France the ripening is carried out in natural caves and in artificial caves hewn from the limestone. Water trickling down through crevices cools the air and at the same time nearly saturates it. The flow of air through the caves is regulated in order to obtain the desired temperature and humidity. In the United States, Roquefort-type cheese is made in curing rooms having regulated relative

humidity and temperature; in caves; and, in one instance at least, in a mine shaft.

Holes are punched into the curd to facilitate the development of the mold throughout the cheese. Although aerobic, *P. roqueforti* will grow with a minimum supply of air. After the mold has developed to the desired extent, the cheese is wrapped in tinfoil and stored at approximately 40°F. (4.44°C.), at which temperature the enzymes of the mold are active, but the mold growth is inhibited.

The pungent taste of Roquefort is due in part to the action of lipase, a fat-splitting enzyme, which liberates caproic, caprylic, and capric acids from the fat.

Roquefort Cheese from Cow's Milk.—Roquefort cheese has been manufactured on a commercial basis from cow's milk since 1918. Roquefort cheese made from cow's milk is, usually, of a more yellow color than the cheese made from sheep's milk.

Other Cheeses.—Brief descriptions and analyses of many varieties of cheese will be found in *Bulletin 608* of the U.S. Department of Agriculture.

For a full discussion of the various cheese-making processes, the reader is referred to the publications cited in the bibliography at the end of the chapter

The U.S. Department of Agriculture has adopted the following definitions and standards for cheese.[⁶]

WHOLE MILK CHEESE

Cheddar Cheese, American Cheese, American Cheddar Cheese.—The cheese made by the Cheddar process from heated and pressed curd obtained by the action of rennet on whole milk. It contains not more than 39 per cent of water, and, in the water-free substance, not less than 50 per cent of milk fat

Pineapple Cheese.—The cheese made by the pineapple Cheddar cheese process from pressed curd obtained by the action of rennet on whole milk. The curd is formed into a shape resembling a pineapple, with characteristic surface corrugations, and during the ripening period the cheese is thoroughly coated and rubbed with a suitable oil, with or without shellac. The finished cheese contains, in the water-free substance, not less than 50 per cent of milk fat.

Brick Cheese.—The quick-ripened cheese made by the brick-cheese process from pressed curd obtained by the action of rennet on whole milk. It contains, in the water-free substance, not less than 50 per cent of milk fat

Stilton Cheese.—The cheese made by the Stilton process from unpressed curd obtained by the action of rennet on whole milk, with or without added cream During the ripening process a special blue-green mold develops, and the cheese thus acquires a marbled or mottled appearance in section

Gouda Cheese.—The cheese made by the Gouda process from heated and pressed curd obtained by the action of rennet on whole milk. The rind is colored with saffron. The finished cheese contains, in the water-free substance, not less than 45 per cent of milk fat.

Neufchâtel Cheese.—The cheese made by the Neufchâtel process from unheated curd obtained by the combined action of lactic fermentation and rennet on whole milk. The curd, drained by gravity and light pressure, is kneaded or worked into a butter-like consistence and pressed into forms for immediate consumption or for ripening. The finished cheese contains, in the water-free substance, not less than 50 per cent of milk fat.

Cream Cheese.—The unripened cheese made by the Neufchâtel process from whole milk enriched with cream. It contains, in the water-free substance, not less than 65 per cent of milk fat.

Roquefort Cheese.—The cheese made by the Roquefort process from unheated, unpressed curd obtained by the action of rennet on the whole milk of sheep, with or without the addition of a small proportion of the milk of goats. The curd is inoculated with a special mold (Penicillium roqueforti) and ripens with the growth of the mold. The fully ripened cheese is friable and has a mottled or marbled appearance in section.

Gorgonzola Cheese.—The cheese made by the Gorgonzola process from curd obtained by the action of rennet on whole milk. The cheese ripens in a cool, moist atmosphere with the development of a blue-green mold and thus acquires a mottled or marbled appearance in section.

WHOLE MILK OR SKIM MILK CHEESE

Edam Cheese.—The cheese made by the Edam process from heated and pressed curd obtained by the action of rennet on whole milk or on partly skimmed milk. It is commonly made in spherical form and coated with a suitable oil and a harmless red coloring matter.

Swiss Cheese.—The cheese made by the Emmenthaler process from heated and pressed curd obtained by the action of rennet on whole milk or on partly skimmed milk. It is ripened by special gas-producing bacteria, causing characteristic "eyes" or holes. The finished cheese contains, in the water-free substance, not less than 45 per cent of milk fat.

Camembert Cheese.—The cheese made by the Camembert process from unheated, unpressed curd obtained by the action of rennet on whole milk or on slightly skimmed milk. It is ripened by the growth of a special mold (Penicillium camemberti) on the outer surface. The finished cheese contains, in the water-free substance, not less than 45 per cent of milk fat.

Brie Cheese.—The cheese made by the Brie process from unheated, unpressed curd obtained by the action of rennet on whole milk, on milk with added cream, or on slightly skimmed milk. It is ripened by the growth of a special mold on the outer surface.

Parmesan Cheese.—The cheese made by the Parmesan process from heated and hard-pressed curd obtained by the action of rennet on partly skimmed milk. The cheese, during the long ripening process, is coated with a suitable oil.

Cottage Cheese.—The unripened cheese made from heated or unheated, separated curd obtained by the action of lactic fermentation or rennet, or a combination of the two, on skimmed milk, with or without the addition of buttermilk. The drained curd may be enriched with cream, and salted or otherwise seasoned.

PASTEURIZED CHEESE

Pasteurized Cheese, Pasteurized-blended Cheese.—The pasteurized product made by comminuting and mixing, with the aid of heat and water, one or more lots of cheese into a homogeneous, plastic mass. The unqualified name "pasteurized cheese," "pasteurized-blended cheese," is understood to mean pasteurized Cheddar cheese, pasteurized-blended Cheddar cheese, and applies to a product which conforms to the standard for Cheddar cheese. Pasteurized cheese, pasteurized-blended cheese, bearing a varietal name is made from cheese of the variety indicated by the name and conforms to the limits for fat and moisture for cheese of that variety

PROCESS CHEESE

Process Cheese.—The modified cheese made by comminuting and mixing one or more lots of cheese into a homogeneous, plastic mass, with the aid of heat, with or without the addition of water, and with the incorporation of not more than 3 per cent of a suitable emulsifying agent The name "process cheese" unqualified is understood to mean process Cheddar cheese, and applies to a product which contains not more than 40 per cent of water and, in the water-free substance, not less than 50 per cent of milk fat. Process cheese qualified by a varietal name is made from cheese of the variety indicated by the name, and conforms to the limits for fat and moisture for cheese of that variety.

References on Cheeses

Decker, J W : "Cheese Making," 5th ed , Mendota Book Co , Madison, Wis , 1909
Doane, C F , and H W Lawson (revised by K J Matheson): Varieties of Cheese, Descriptions and Analyses, *U S Dept Agr , Bull* 608, February, 1932
Eckles, C H , W B Combs, and H Macy: "Milk and Milk Products," 3d ed , McGraw-Hill Book Company, Inc , New York, 1943
Girard, J D , A Missky, J H Baker, and V Pascale Identification of Roquefort Cheese, *Ind Eng Chem*, **29**: 1167–1171 (1937)
Gurl, C M : Making American Cheese on the Farm, *U S Dept Agr , Farmers' Bull* 1191, June, 1921
Golding, N S : "Methods Used to Increase Blue Mold Growth in Cheese," pp 113 118, D W Hammer Panegyric, C B Lane, Dairy Industry Department, Iowa State College, Ames, Iowa, 1937
Hussong, A T : "Molds, Yeasts, and Actinomycetes," John Wiley & Sons, Inc , New York, 1930
Hull, R S , and A C Merrill The Manufacture of Cheese from Soft-curd and Hard-curd Milks, *Utah State Agr Expt Sta , Bull* 276, 1932
Kraft Phenix Cheese Corporation "The Romance of Cheese," 1936
Lucas, P S : Cottage Cheese, *Mich State Coll Agr Expt Sta , Cir Bull* 97, April, 1931

MARQUARDT, J. C.: The Salting and Cooking of Curds in the Manufacture of Several Varieties of Cheeses, *N.Y. State Agr. Expt. Sta., Bull.* 670, 1936.

————: Methods for Determining Salt in Various Cheeses, *N.Y. State Agr. Expt Sta., Tech. Bull.* 219, September, 1938.

MARRE, E.: "Le Roquefort," E. Carrère, Rodez, France, 1906.

MATHESON, K J : Neufchâtel and Cream Cheese: Farm Manufacture and Use, *U.S. Dept. Agr., Farmers' Bull.* 960, October, 1934.

PRESCOTT, S. C., and B. E. PROCTOR: "Food Technology," McGraw-Hill Book Company, Inc , New York, 1937.

PRICE, W. V.: The Manufacture of Cheddar Cheese from Milk Pasteurized by the Holder Method, *Cornell Univ. Agr. Expt. Sta , Mem* 105, April, 1927.

ROGERS, L A.: Packaging American Cheeses in Cans, *Food Industries,* 6: 308 (1934).

———— (associates of): "Fundamentals of Dairy Science," 2d ed., Reinhold Publishing Corporation, New York, 1935.

————: Science and Art of Cheesemaking. *Scientific Monthly,* 42: 437–443 (1936).

SHERMAN, H. C.: "Food Products," 4th ed., The Macmillan Company, New York, 1948.

THOM, C : Camembert Cheese Problems in the United States, *U.S. Dept. Agr , Bur. Agr. Ind , Bull* 115, 1909.

————: "The Penicillia," The Williams & Wilkins Company, Baltimore, 1930

———— and W W FISK: "The Book of Cheese," The Macmillan Company, New York, 1918.

WINTON, A L., and K B WINTON: "The Structure and Composition of Foods," Vol III, "Animal Products," John Wiley & Sons, Inc., New York, 1937.

CHAPTER XXI

THE PROPIONIC ACID FERMENTATION

Historical.—In 1841 Nöllner[1] isolated a "Pseudo-Essigsäure" (pseudo-acetic acid) from the decomposition products of tartaric acid. This substance is believed to have been largely propionic acid (CH_3CH_2COOH)

Fitz, in a series of articles on fermentation,[2] discussed the morphology and biochemical activities of the bacteria producing propionic acid from calcium malate and from calcium lactate.

Orla-Jensen[3] (1898) carried out research concerning the formation of eyes in Emmenthaler cheese. Later von Freudenreich and Orla-Jensen[4] reported the first description of the isolation of microorganisms causing the propionic acid fermentation They studied the propionic acid bacteria rather extensively, especially in relation to cheese

As the result of investigations carried out to discover the reasons why Emmenthaler cheese manufactured in the United States was lacking in characteristic flavor and why the eye formation was deficient or abnormal, Sherman and Shaw[5] supplied much important information in respect to the propionic acid bacteria, for example, they demonstrated the relationship between the propionic acid bacteria and the production of a high-grade Emmenthaler cheese. Sherman (1921) described the use of pure cultures of propionic acid bacteria, *Bacterium acidi-propionici*, to ensure the production of Emmenthaler cheese with characteristic flavor and with normal eye development. This organism did not suppress the growth of undesirable bacteria, however. Cultures of *Lactobacillus bulgaricus* have long been used in cheese making to inhibit the development of the wrong types of bacteria

Whittier and Sherman[6] studied the factors affecting the propionic acid

[1] Nöllner, C., *Ann*, **38**: 299 (1841)
[2] Fitz A., *Ber*, **9**: 1348 (1876) **10** 276 (1877) **11**: 42, 1890 (1878) **12**, 474 (1879), **13** 1309 (1880) **14**: 1084 (1881) **15** 867 (1882) **16**, 844 (1883); **17**: 1188 (1884)
[3] Orla-Jensen, S , *Centr Bakt Parasitenk* , Abt **II**, **4**: 217 263, 325 (1898)
[4] Freudenreich, E von und Orla-Jensen, S , *Centr Bakt Parasitenk* Abt **II**, **17** 529 (1906)
[5] Sherman, J M and R H Shaw *Jour Biol Chem* **56** 695 (1923)
[6] Whittier, E O and J M Sherman *Ind Eng Chem* **15**: 729 (1923)
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fermentation, and, with *Albus*, the rates of fermentation of lactose, galactose, glucose, sucrose, and maltose.[1]

Virtanen[2] has studied the mechanism of the propionic acid fermentation.

Van Niel's[3] dissertation on the propionic acid bacteria was published in 1928. This comprehensive monograph will prove to be of much value to anyone interested in the propionic acid bacteria.

Werkman, Wood, Stone, and their associates have carried out considerable research on the propionic acid fermentation, particularly in respect to the mechanism of the fermentation. Some of their work will be discussed later.

Tatum, Peterson, and their coworkers[4] have studied the effect of growth factors on propionic acid bacteria.

Factors Affecting the Fermentation. *The Organism.*—Propionic acid bacteria, in general, may be characterized as Gram-positive, catalase positive, nonsporeforming, nonmotile, facultative aerobes.

Van Niel[5] lists eight main species (with their synonyms) on the basis of morphological, cultural, and biochemical differences:

Propionibacterium freudenreichi (*Bacterium acidi propionici a* von Freudenreich and Orla-Jensen, etc.)
P. jensenii (*Bact. acidi propionici b* von Freudenreich and Orla-Jensen)
P. petersonii (*Bact. acidi propionici c* Trolli Petersson)
P. shermanii (*Bact. acidi propionici d* Sherman)
P. pentosaceum (*Bacillus acidi propionici* von Freudenreich and Orla-Jensen)
P. rubrum (*Bact. acidi propionici* var. *rubrum*   Thoeni and Allemann)
P. thoenii (*Bact. acidi propionici* var. *rubrum.*   Thoeni et Allemann)
P. technicum

The following additions to this list have been suggested:

P. raffinosaceum   Werkman and Kendall[6]
P. arabinosum   Hitchner[7]
P. zeae   Hitchner[7]

[1] Whittier, E. O., J. M. Sherman, and W. R. Albus, *Ind. Eng. Chem.*, 76: 122 (1924).

[2] Virtanen, A. I., *Soc. Sci. Fennica, Commentationes Phys. Math.*, 1 (No. 36), 1 (1923), 2 (No. 20), 1 (1925).

[3] Van Niel, C. B., "The Propionic Acid Bacteria," Technische Hoogeschool, Delft, September, 1928.

[4] Tatum, E. L., W. H. Peterson, and E. B. Fred, *Jour. Bact.*, 32: 157 (1936). Tatum, E. L., H. G. Wood, and W. H. Peterson, *Jour. Bact.*, 32: 167 (1936); Snell, E. E., F. M. Strong, and W. H. Peterson, *Jour. Bact.*, 38: 293 (1939).

[5] Van Niel, *loc. cit.*

[6] Werkman, C. H., and S. E. Kendall, *Iowa State Coll. Jour. Sci.*, 6: 17 (1931).

[7] Hitchner, E. R., *Jour. Bact.*, 23: 40 (1932), 28: 473 (1934).

P. technicum has the ability to ferment starch, dextrin, and glycogen—other previously described propionic acid bacteria did not possess this ability. *P. thoenii* produces propionic acid and acetic acid from a glucose medium containing yeast extract in a molecular ratio of approximately 5:1, according to Van Niel.

Propionic acid bacteria may be isolated from a number of sources: milk, cheeses, and other dairy products; silage; soil; the excreta of cattle; and other sources.

The Carbon Source.—A large number of raw materials have been utilized as sources of carbon by different species of *Propionibacterium*. Some materials fermented include: lactose, sucrose, maltose, glucose, raffinose, arabinose, xylose, glycogen, dextrin, and starch; lactic, tartaric, and quinic acids; glycerol and mannitol; and proteins, protein derivatives, and fats. Lactose and low-priced carbohydrates would undoubtedly be used in the industrial production of propionic acid.

The Nitrogen Source.—Propionic acid bacteria may utilize several nitrogen-containing compounds. One of the most satisfactory nitrogen sources is yeast extract, at a concentration of approximately 0.4 per cent. Peptones, whey, and corn meal may be utilized, especially in the association of other bacteria, such as *Proteus mirabilis*,[1] *Streptococcus lactis*,[2] or *Lactobacillus casei*.[2]

Sherman suggests that the increased production of propionic acid resultant from the association of bacteria may be due to the fact that lactic acid is more readily utilized than lactose by the propionic acid bacteria. Van Niel suggests that the stimulating effect may be due to an alteration in the nitrogenous compounds of the medium.

Tatum and his associates[4] state that the propionic acid bacteria may be able to utilize ammonia as the only source of nitrogen when suitable stimulatory substances are present. They suggest that yeast extract and other complex nitrogen sources may be effective because they contain nonnitrogenous growth factors in addition to available nitrogen.

The nitrogen source is of much importance for it influences the rate and the completeness of fermentation, also the ratio of propionic acid to acetic acid.

Growth Factors—It has been stated repeatedly in some of the earlier literature that a complex source of nitrogen was essential for the growth of propionic acid bacteria. Recent research has indicated that growth factors rather than complex nitrogen sources are most important.

[1] Van Niel, *loc. cit.*
[2] Sherman, J. M., and R. H. Shaw, *Jour. Gen. Physiol.* **3**: 657 (1921).
[4] Tatum, Wood, and Peterson, *loc. cit.*

According to Wood and his associates,[1] amino acids are beneficial but not essential for the growth of propionic acid bacteria.

Wood and his coworkers[2] obtained an ether-soluble factor from yeast extract, which was indispensable for the growth of all the cultures of propionic acid bacteria tested on a synthetic medium that contained ammonium sulphate as the nitrogen source. This factor has been found in potato extract, corn extract, corn steep, and liver extract, in addition to yeast extract. It is a nonvolatile acid, soluble in chloroform, benzene, ether, and xylene but almost insoluble in petroleum ether. It may be adsorbed on Norit and eluted with acid-alcohol. The factor is *not* replaced by a mixture containing vitamin B_1, nicotinic acid, pimelic acid, uracil, beta-alanine, and pantothenic acid.

Vitamin B_1 (thiamin) stimulated the growth of propionic acid bacteria, especially in the presence of amino acids. But not all *propionic acid bacteria require Vitamin B_1 for vigorous growth, according to* Tatum and his associates[3]

Riboflavin,[4] in a concentration of 0.05 gamma per cubic centimeter, stimulated the growth of propionic acid bacteria in a medium containing ammonium sulphate.

For a further discussion of this subject the reader is urged to consult the various papers on growth factors that are cited at the end of *this* chapter.

The pH.—Most favorable results are obtained when the pH is adjusted to 6.8 to 7.2, a pH of 7.0 being *usually preferred.*

The Temperature.—The optimum temperature for fermentation is approximately 30°C.

Duration of Fermentation.—The fermentation *normally requires* 7 to 12 days, but, by adapting the fermentation to a *semicontinuous* basis, Van Niel demonstrated that the fermentation time may be considerably reduced.

Composition of Some Culture Media.—Van Niel[5] used a culture medium containing yeast extract and 2 per cent sodium lactate, adjusted to a pH of 7 in some of his researches. Another medium used by him contained yeast extract, 2 per cent glucose, and 2 per cent calcium carbonate.

[1] Wood, H. G., A. A. Andersen, and C. H. Werkman, *Jour. Bact.*, 36: 201 (1938).
[2] Wood, H. G., E. L. Tatum, and W. H. Peterson, *Jour. Bact.*, 33: 227 (1937)
[3] Tatum, E. L., H. G. Wood, and W. H. Peterson, *Biochem. Jour.*, 30: 1898 (1936).
[4] Wood, H. G., A. A. Andersen, and C. H. Werkman, *Proc. Soc. Exptl. Biol. Med.*, 36: 217 (1937); Lava, V. G, R Ross, and K. C. Blanchard, *Philippine Jour. Sci.*, 59: 493 (1936).
[5] Van Niel, *loc. cit.*

Tatum, Peterson, and Fred[1] have cultured the organisms on a medium containing 1 per cent malt sprouts, 1 per cent glucose, and calcium carbonate.

A medium consisting of 5 g. of lactose, 5 g. of calcium carbonate, and 1 g. of dried yeast in 100 cc. of water, with the pH adjusted to approximately 7, was used by Whittier and Sherman[2] under certain conditions.

A basal medium containing 1 per cent glucose, 0 6 per cent sodium lactate, 0 3 per cent ammonium sulphate, and Speakman's inorganic salts in half concentration[3] (0 25 g. K_2HPO_4, 0.25 g. KH_2PO_4, 0 1 g. $MgSO_4\cdot7H_2O$, 0.005 g. NaCl, 0.005 g. $FeSO_4\cdot7H_2O$, 0.005 g. $MnSO_4\cdot4H_2O$, and 1,000 cc water)[4] was used by Wood and his coworkers in some of their research

Products of the Fermentation.—The main end products of the propionic acid fermentation are propionic acid, acetic acid, and carbon dioxide. Small amounts of succinic acid are frequently produced, while acetylmethylcarbinol is occasionally formed by certain species.

Ratio of Propionic Acid to Acetic Acid.—The ratio of propionic to acetic acid varies according to the species, the nitrogen source, and other factors. In a medium containing glucose and yeast extract, the acids were produced in a ratio of approximately 5.1 by *Propionibacterium thoenii* Under similar conditions the acids were formed in a ratio of about 3 1 by *P rubrum* [5] When using *P. shermanii* (*Bacterium acidi propionici d*), Whittier and Sherman observed a fairly constant ratio of 2 molecules of propionic acid to 1 molecule of acetic acid.[6]

Yields.—Usually more than 75 per cent of the fermented sugar may be accounted for as propionic and acetic acids, while less than 20 per cent is used for carbon dioxide production.

In one experiment, in which the medium contained yeast extract, 2 per cent glucose, and 2 per cent calcium carbonate, Van Niel obtained the following results 13 21 g. glucose fermented; and 8.62 g propionic acid, 1.85 g acetic acid, 1.363 g. carbon dioxide, and 0 45 g. succinic acid produced

Whittier[6] and Sherman have determined the conditions whereby yields of approximately 2.4 lb of propionic acid and 1 lb of acetic acid may be obtained from 5 lb of lactose after a fermentation period of 12 days at 30°C., using a mixed culture of *P shermanii* and *Lactobacillus*

[1] Tatum, Peterson, and Fred, *loc cit*

[2] Whittier and Sherman, *loc cit*

[3] Wood, H G, A A Anderson, and C H Werkman, *Jour Bact*, **36**:201 (1938)

[4] Wood, Tatum, and Peterson, *loc cit*

[5] Van Niel, *loc cit*

[6] Whittier and Sherman, *loc cit*

casei as the inoculum. **Yields of 85 per cent or greater usually required** a fermentation period of 2 weeks or longer.

Uses.—The propionic acid bacteria determine the taste and flavor as well as *the characteristic eye development* in Emmenthaler cheese.

Propionic acid is used in the manufacture of perfumes. Ethyl propionate is a solvent for pyroxylin. The mixture of propionic and acetic acids may be distilled to produce a mixture of acetone, methylethyl ketone, and diethyl ketone,[1] substances which are solvents.

Large quantities of propionic acid could be manufactured industrially by fermentation, provided that a demand arose for the acid.

The Mechanism of the Propionic Acid Fermentation.—Fitz[2] proposed that lactic acid was converted by propionic acid bacteria in accordance with the following equation:

$$
\begin{array}{ccccc}
CH_3 & & CH_2 & & \\
| & & | & & \\
3CHOH & = & 2CH_2 & + CH_3 & + CO_2 + H_2O \\
| & & | & | & \\
COOH & & COOH & COOH & \\
\text{Lactic acid} & & \text{Propionic acid} & \text{Acetic acid} &
\end{array}
$$
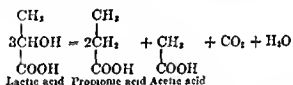
Virtanen,[3] Virtanen and Karström,[4] and Van Niel[5] were of the opinion that two 3-carbon molecules were formed from glucose after phosphorylation, one of these being oxidized to acetic acid and carbon dioxide while two other molecules were being reduced to propionic acid.
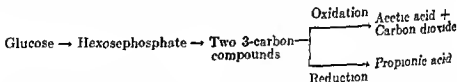
Van Niel proposed that pyruvic acid acted as an intermediate. Virtanen[3] assumed that succinic acid arose in the following manner from glucose.

$$
\begin{array}{ccc}
& CH_2 COOH & CH_3 \\
| & | & | \\
C_6H_{12}O_6 & CH_2 COOH & + CHO + H_2O \\
\text{Glucose} & \text{Succinic acid} & \text{Acetaldehyde}
\end{array}
$$
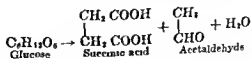
Wood, Stone, and Werkman[6] have proposed the following scheme for the dissimilation of glucose by propionic acid bacteria:

[1] WHITTIER and SHERMAN, *loc. cit.*
[2] FITZ, A., *Ber.*, **11**: 1896 (1878).
[3] VIRTANEN, *loc. cit.*
[4] VIRTANEN, A. I., and H. KARSTRÖM, *Acta Chem. Fennica*, Ser. B. **7**: 17 (1931).
[5] VAN NIEL, *op. cit.*
[6] WOOD, H. G., R. W. STONE, and C. H. WERKMAN, *Biochem. Jour.*, **31**: 319 (1937).

$$C_6H_{12}O_6 \xrightarrow[-H_2O]{+2H_3PO_4} \text{Phosphorylated hexose} \rightarrow \begin{array}{c} CHO \\ | \\ CHOH \\ | \\ CH_2O(PO_3H_2) \\ \text{Triosephosphate} \end{array} \rightarrow \text{Nonreducing material}$$

$$\begin{array}{c} COOH \\ | \\ CHOH \\ | \\ CH_2O(PO_3H_2) \\ \text{Phosphoglyceric acid} \end{array} \xrightarrow[-2H]{+H_2O}$$

$$\begin{array}{c} COOH \\ | \\ C \begin{array}{c} OH \\ OH \end{array} \\ | \\ CH_3 \\ \text{Pyruvic acid hydrate} \end{array} \xrightarrow[-2H]{+H_2O} \begin{array}{c} COOH \\ | \\ CO \\ | \\ CH_3 \\ \text{Pyruvic acid} \end{array} \xleftarrow{-H_3PO_4}$$

$$\begin{array}{c} COOH \\ | \\ CH_3 \\ \text{Acetic acid} \end{array} \xleftarrow[-2H]{+CO_2} \begin{array}{c} COOH \\ | \\ CHOH \\ | \\ CH_3 \\ \text{Lactic acid} \end{array} \xrightarrow{-2H}$$

$$\begin{array}{c} CHO \\ | \\ CO \\ | \\ CH_3 \\ \text{Methylglyoxal} \end{array} \xrightarrow{+H_3PO_4}$$

$$\begin{array}{c} H_3C\ COOH \\ \\ H_3C\ COOH \\ \text{Succinic acid} \end{array} \xrightarrow{-2H}$$

$$\begin{array}{c} COOH \\ | \\ CH_2 \\ | \\ CH_3 \\ \text{Propionic acid} \end{array} \xleftarrow[-H_2O]{+2H}$$

$CO_2 + \text{Synthesis of fermentable unknown compound}$

Some Possible Evidence for Foregoing Schemes.

—Several compounds that may act as intermediates have been isolated or detected in media fermented by normal or dried propionic acid bacteria. Lactic acid has been demonstrated by Foote and his associates[1] and by Fromageot and Tatum.[2] Virtanen and Karström found hexose-monophosphate[3] Methylglyoxal was formed by the action of dried propionic acid bacteria on magnesium hexose phosphate[4] Pyruvic acid was identified in the fermentation of glucose by *P. arabinosum*.[5] The same two men have isolated propionaldehyde from the fermentation of glycerol[5] In 1936, Stone and Werkman isolated phosphoglyceric acid from a fermentation medium containing glucose, toluene, and sodium fluoride

[1] Foote, M., J. B. Urro, and W. H. Peterson, *Centr. Bakt. Parasitenk.*, Abt. II, 82 379 1930.

[2] Fromageot, C. and L. L. Tatum, *Biochem. Zeit.*, 267: 360 (1933).

[3] Virtanen and Karström, *loc. cit.*

[4] Pett, L. B., and A. M. Wynne, *Trans. Roy. Soc. Can.*, 27: 119 (Sect V) (1933)

[5] Wood, H. G. and C. H. Werkman, *Biochem. Jour.*, 28: 745 (1934).

[5] Wood, H. G. and C. H. Werkman, *Proc. Soc. Exptl. Biol. Med.*, 31:930 (1934)

References on Propionic Acid Bacteria

ERB, C., H. G. WOOD, and C. H. WERKMAN: The Aerobic Dissimilation of Lactic Acid by the Propionic Acid Bacteria, *Jour. Bact.*, 31: 595 (1936).

FITZ, A.: Über die Gährung des Glycerins, *Ber.* 9: 1348 (1876).

———: Über Schizomyceten—Gährungen. II. Glycerin, Mannit, Stärke, Dextrin, *Ber.*, 10: 276 (1877).

———: Über Schizomyceten—Gährungen III, *Ber.*, 11: 42 (1878).

———: Über Spaltpilzgährungen, *Ber.*, 11: 1890 (1878); 12: 474 (1879); 13: 1309 (1880).

———: II Über Doppelsalze der niedrigen Fettsäuren, *Ber.*, 14: 1084 (1881).

———: Über Spaltpilzgährungen, *Ber.*, 15: 867 (1882); 16: 844 (1883); 17: 1188 (1884).

FOOTE, M , E. B. FRED, and W. H. PETERSON: The Fermentation of Pentoses by Certain Propionic Acid Bacteria, *Centr. Bakt. Parasitenk.*, Abt. II, 82: 379 (1930).

FREUDENREICH, E. VON, und ORLA-JENSEN: Über die Emmentalerkäse stattfindende Propionsäuregärung, *Centr. Bakt. Parasitenk.*, Abt. II, 17: 529 (1906).

FROMAGEOT, C., und E. L. TATUM: Über einen Aktivator des Stoffwechsels der Propionsaurebakterien, *Biochem. Zeit.*, 267: 360 (1933).

HITCHNER, E. R.: Some Physiological Characteristics of the Propionic-acid Bacteria, *Jour. Bact.*, 28: 473 (1934)

KOSER, S A., and F. SAUNDERS: Accessory Growth Factors for Bacteria and Related Microorganisms, *Bact. Rev.*, 2: 117 (1938).

LAVA, V G., R. ROSS, and K C BLANCHARD: Is Vitamin B₁ the Accelerating Factor in the Fermentation of Sugar by Propionic Acid Organisms, *Philippine Jour. Sci*, 59: 493 (1936).

MAY, O. E., and H. T. HERRICK: Production of Organic Acids from Carbohydrates by Fermentation, *U.S Dept. Agr., Circ.* 216, May, 1932

ORLA-JENSEN, S.: Studien über die Lochbildung in den emmanthaler Kasen, *Centr Bakt. Parasitenk*, Abt II, 4: 217, 265, 325 (1898).

———: "Dairy Bacteriology," P. Blakiston's Son & Company, Inc , Philadelphia, 1931.

PETT, L. B , and A. M. WYNNE: The Metabolism of Propionic Acid Bacteria I The Degradation of Phosphoric Acid Esters by *Propionibacterium Jensen* (van Niel), *Trans. Roy. Soc. Can.*, 27: 119 (Sect. V): (1933).

SHAW, R. H., and J. M. SHERMAN: The Production of Volatile Fatty Acids and Carbon Dioxide by Propionic Acid Bacteria with Special Reference to Their Action in Cheese, *Jour. Dairy Sci* , 6: 303 (1923).

SHERMAN, J. M.: The Cause of Eyes and Characteristic Flavor in Emmenthal or Swiss Cheeses, *Jour. Bact.* , 6: 379 (1921).

———: The Use of Bacterial Cultures for Controlling the Fermentation in Emmenthal Cheese, *Proc. World's Dairy Congress*, 1: 287 (1923).

——— and R. H SHAW: Associative Bacterial Action in the Propionic Acid Fermentation, *Jour. Gen. Physiol.*, 3: 657 (1921).

——— and ———: The Propionic Fermentation of Lactose, *Jour. Biol. Chem*, 56: 695 (1923).

SNELL, E E , F. M. STRONG, and W. H. PETERSON: Growth Factors for Bacteria VIII. Pantothenic and Nicotinic Acids as Essential Growth Factors for Lactic and Propionic Acid Bacteria, *Jour. Bact*, 38: 293 (1939).

Stone, R. W., H. G. Wood, and C. H. Werkman: Activities of the Lower Fatty Acids by Propionic Acid Bacteria, *Jour. Bact.*, 30: 652 (1935).

————, and ————: Activation of the Lower Fatty Acids by Propionic Acid Bacteria, *Biochem. Jour.*, 30: 621 (1936).

Tatum, E. L., W. H. Peterson, and E. B. Fred: Essential Growth Factors for Propionic Acid Bacteria I. Sources and Fractionation, *Jour. Bact.*, 32: 157 (1936)

————, H. G. Wood, and W H Peterson: Essential Growth Factors for Propionic Acid Bacteria II Nature of the Neuberg Precipitate Fraction of Potato: Replacement by Ammonium Sulphate or Certain Amino Acids, *Jour. Bact.*, 32: 167 (1936).

————, ————, and ———— Growth Factors for Bacteria V Vitamin B_1, a Growth Stimulant for Propionic Acid Bacteria, *Biochem. Jour.*, 30: 1898 (1936).

Van Niel, C B "The Propionic Acid Bacteria," Thesis, Technische Hoogeschool, Delft, September, 1928.

Virtanen, A. I Propionic Acid Fermentation, *Soc Sci. Fennica, Commentationes Phys Math*, 1 (No 36): 1 (1923), 2 (No 20): 1, (1925)

———— and H Karström *Acta Chem Fennica, Ser B*, 7: 17 (1934).

Werkman, C H, and R W. Brown: The Propionic Acid Bacteria. II. Classification, *Jour Bact*, 26: 393 (1933)

————, R M Hixon, E I Fulmer, and C H Rayburn The Production of Propionic Acid from Pentoses by *Propionibacterium pentosaceum*, *Proc. Iowa Acad Sci*, 36: 111 (1929)

Whistler, I O, and J M Sherman: Propionic Acid and Ketones from Whey, *Ind. Eng Chem*, 16: 729 (1923)

———, ———, and W R Aldus The Rates of Fermentation of Sugars by the Propionic Organisms, *Ind Eng Chem*, 16: 122 (1924)

Wood, H G, A A Anderson, and C H Werkman Growth Factors for Propionic and Lactic Acid Bacteria, *Proc Soc Expt Biol Med*, 36: 217 (1937)

————, and ———— Nutrition of the Propionic Acid Bacteria, *Jour. Bact*, 36: 201 (1938)

—— R W Stone, and C H Werkman The Intermediate Metabolism of the Propionic Acid Bacteria, *Biochem Jour*, 31: 349 (1937)

, E L Tatum, and W H Peterson Growth Factors for Bacteria IV An Acidic Ether-soluble Factor Essential for Growth of Propionic Acid Bacteria, *Jour Bact*, 33: 227 (1937)

—— and C H Werkman The Propionic Bacteria on the Mechanism of Glucose Dissimilation, *Jour Biol Chem*, 105: 63 (1934)

—— and —— CHR Pyruvic Acid in the Dissimilation of Glucose by the Propionic Acid Bacteria, *Biochem Jour*, 29: 745 (1934)

—— and —— The Isolation and Possible Intermediary Role of Formaldehyde in the Propionic Acid Fermentation, *Jour Bact*, 30: 652 (1935)

—— and —— X The Utilization of CO_2 in the Dissimilation of Glycerol by the Propionic Acid Bacteria, *Biochem Jour*, 30: 48 (1936)

—— and —— LXXXVIII Mechanism of Glucose Dissimilation by the Propionic Acid Bacteria, *Biochem Jour*, 30: 618 (1936)

—— and —— The Utilization of Carbon Dioxide by the Propionic Acid Bacteria, *Biochem Jour*, 32: 1262 (1938)

W F Wiggert and C H Werkman The Fermentation of Phosphate Esters by the Propionic Acid Bacteria, *Enzymologia*, 2: 373 (1936)

Some Patents

SHERMAN, J. M.: Propionic Acid Fermentation by the Use of Mixed Strains of Propionic Bacteria, U.S. Patent 1,865,146, June 28, 1932.

————: Method of Accelerating the Propionic Acid Fermentation, U.S. Patent 1,910,130, May 23, 1933.

STILES, H. R., and P. W. WILSON: Production of Propionic Acid, U.S. Patent 1,932,755, Oct. 31, 1933.

STILES, H. R.: Propionic Acid Fermentation of Fructose-containing Mashes, U S Patent 1,946,447, Feb. 6, 1934.

WILSON, P. W.: Propionic Acid Fermentation, U.S. Patent 1,898,329, Feb. 21, 1933

WOODRUFF, C., and P W. WILSON: Propionic Acid Fermentation, U.S Patent 1,875,401, Sept. 6, 1932.

THE PRODUCTION AND PROPERTIES OF 2,3-BUTANEDIOL

2,3-Butanediol ($CH_3 \cdot CHOH \cdot CHOH \cdot CH_3$) is well known as 2,3-butylene glycol. It is somewhat less commonly known as 2,3-dihydroxybutane or dimethylethylene glycol.

The demand for synthetic rubber in World War II greatly stimulated research on the production and properties of 2,3-butanediol in this country and in Canada, since it could be converted to 1,3-butadiene, a substance used in the production of rubber of the Buna type. Since insufficient knowledge was available concerning the production of 2,3-butanediol and conversion methods at the time the synthetic rubber industry was being established, it was necessary to manufacture butadiene from other raw materials, such as ethyl alcohol. During the war years and since, a great deal of information has been obtained regarding production methods, particularly on laboratory and pilot-plant scales. In addition, it has been shown that derivatives may be readily prepared from the 2,3-butanediols and that there are many potential uses for these, such as for antifreeze agents, solvents, softeners, and plastics.

Historical.— The literature on this subject has been reviewed by Ledingham, Adams, and Stanier;[1] and by Underkofler and Fulmer.[2]

The first significant reports concerning the *Aerobacter* fermentation were those of Harden and Walpole (1906); Walpole (1911); and Scheffer (1928). Following these there appeared a number of publications from the Iowa State College, among which may be mentioned those of Breden (1930), Breden and Fulmer (1931); Fulmer, Christensen, and Kendall (1933); Kendall (1934), Chappell (1935); Porter, McCloskey, and Levine (1937), Silverman and Werkman (1941), Stahly and Werkman (1942), and Underkofler and Fulmer (1948).

Reports concerning the fermentation brought about by *Aerobacillus polymyxa*[3] have issued largely from Canada, although the first significant

[1] Ledingham, G. A., G. A. Adams, and R. Y. Stanier, Can. Jour. Research, F, **23** 48 (1945)

[2] Underkofler, L. A., and E. I. Fulmer, Ia. Western Lab. Commun. **11** (No 32) 41 (1948)

[3] This organism is classified as *Bacillus polymyxa* in the sixth edition of "Bergey's Manual of Determinative Bacteriology

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CHAPTER XXII

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[1] LEDINGHAM, G A, G A ADAMS, and R Y STANIER, Can Jour Research, F, 23: 48 (1945)

[2] UNDERKOFLER, L A, and J J FULMER, Wallerstein Labs Commun., 11 (No 32) 41 (1948)

[3] This organism is classified as *Bacillus polymyxa* in the sixth edition of "Bergey's Manual of Determinative Bacteriology"

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information regarding this fermentation was supplied by Donker (1926) in Holland, who showed that 25 per cent of 2,3-butanediol and 19 per cent of ethanol were obtained in the fermentation of glucose. A large number of reports has been published since 1914, in particular by Ledingham, Neish, Adams, Stanier, Rose, Leslie, Fratkin, and their associates of the National Research Council of Canada; and also by Katznelson, Lochhead, and fellow workers of the Division of Bacteriology and Dairy Research, Canada Department of Agriculture.

During World War II, a considerable amount of coordinated research was carried out simultaneously by personnel of the Northern Regional Research Laboratory of the U. S. Department of Agriculture; the National Research Council and the Department of Agriculture in Canada; the Iowa State College; the University of Wisconsin; Joseph E. Seagrams and Sons, Inc.; the Commercial Solvents Corporation; Schenley Distillers Corp.; and by members of other laboratories. In the following paragraphs reference is made to the results of some of the research carried out by these organizations and other individuals.

The Fermentations.—A number of species or strains of bacteria classified in the genera *Aerobacter, Aerobacillus, Aeromonas, Serratia,* and *Bacillus* possess the ability to produce 2,3-butanediol. The fermentations brought about by strains of *Aerobacter aerogenes* and *Aerobacillus polymyxa* (Pruzmowski) Migula have been studied intensively and appear to be the most important ones. However, considerable interest has been shown in the fermentations produced by *Aeromonas hydrophila, Bacillus subtilis* (Ford's strain) and *Serratia marcescens.*

Products of the Fermentations.—The products formed during each type of fermentation vary qualitatively and quantitatively, depending on the strain of organism used, the media, and the conditions of fermentation. However, in each instance, there are produced 2,3-butanediol, acetoin (acetylmethylcarbinol), ethanol, carbon dioxide, acids, and occasionally other substances. These will be considered in detail in the following discussion.

Forms of 2,3-Butanediol.—There are three stereoisomeric forms of 2,3-butanediol, or 2,3-butylene glycol, all of which are produced by bacterial fermentation. These are the dextro, levo, and meso forms, the structural formulas of which are shown below:

d-(−)-2,3-Butanediol   l-(+)-2,3-Butanediol   meso-2,3-Butanediol
(Levorotatory form)   (Dextrorotatory form)   (Optically inactive form)
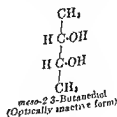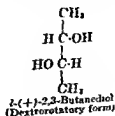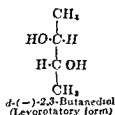
A mixture of the dextrorotatory and meso forms of 2,3-butanediol is produced by strains of *Aerobacter aerogenes*.

The levorotatory form of 2,3-butanediol is produced characteristically by *Bacillus polymyxa* (Prazmowski) Migula. Ward and his associates[1] identified D-(-)-2,3-butanediol as the chief substance formed by *B. polymyxa* in grain mashes. This substance possessed a specific rotation slightly in excess of $-13.0°$. According to Neish,[2] the D-(-)-2,3-butanediol produced by *B. polymyxa* is a pure isomer, with the optical rotation $[a]_D^{25} = -13.34°$.

A mixture of the levorotatory and meso forms of 2,3-butanediol is produced by *Aeromonas hydrophila*. According to Stanier and Adams,[3] the levorotatory form preponderates, based on the optical rotation $[a]_D^{25} = -0.97$.

Bacillus subtilis (Ford's Strain) produces a mixture of the levorotatory and meso forms of 2,3-butanediol in approximately equal quantities, according to Neish, Blackwood, and Ledingham[4][5] The optical rotation $[a]_D^{15}$ equals $-5.0°$. The refractive index is 1 4340 at 26°C., while the boiling point is 181 to 182°C. at 760 mm pressure.

The 2,3-butanediol produced in the *Serratia marcescens* fermentation is a mixture of the meso- and dextrorotatory forms,[6] but the amount of the latter is only about 2 per cent of the total.

Production of 2,3-Butanediol.—As already indicated, 2,3-butanediol is produced in reasonable yields by strains of several different groups of bacteria. These will be considered individually since production methods vary.

1 The Aerobacter aerogenes Fermentation.—This fermentation was first studied by Harden and Walpole[7] in 1906. The organism *B. lactis aerogenes* (*Aerobacter aerogenes*) was grown under anaerobic conditions on a medium containing 1 per cent Witte peptone, 2 per cent glucose (or mannitol), and chalk. Later (1911) Walpole[8] investigated the fermenta-

[1] WARD, G L , O G PETTIJOHN, L B LOCKWOOD, and R D COGHILL, *Jour. Am Chem Soc*, 66 541 (1944).

[2] NEISH, A C , *Can Jour Research*, B, 23 10 (1945)

[3] STANIER, R Y , and G A ADAMS, *Biochem Jour* 38 168 (1944)

[4] NEISH, A C , A C BLACKWOOD, and G A LEDINGHAM, *Science*, 101 215 (1945)

[5] NEISH, A C , A C BLACKWOOD, and G A LEDINGHAM, *Can Jour Research*, B, 23 290 1945)

[6] NEISH, A C , A C BLACKWOOD, F M ROBERTSON, an I G A LEDINGHAM, *Can Jour Research*, B, 25 65 (1947)

[7] HARDEN, A , and G S WALPOLE *Proc Roy Soc (London , Series B, 77 399 1906.

[8] WALPOLE, G S , *Proc Roy Soc (London) Series* B, 83 272 (1911)

tion further. In 1928, Scheffer[1] carried out research on the production of 2,3-butanediol by *A. aerogenes* and related organisms

Fulmer, Christensen, and Kendall[2] investigated the effect of sucrose concentration on the production of 2,3-butanediol by species of *Aerobacter*, particularly *A. pectinovorum* (a variety of *A. aerogenes*). The medium contained the following materials per 100 ml.: 0 250 g. of ammonium chloride, 0.150 g. of potassium monophosphate, 0.150 g of calcium chloride, and 0.20 g. of magnesium sulphate, plus different quantities of sucrose. The pH was adjusted to an optimum of 6.0 with sodium carbonate. Sucrose in concentrations up to and including 8 per cent was entirely fermented. As the concentrations were increased above 8 per cent, the per cent of sucrose fermented dropped. At a concentration of 8 per cent, 47 g. of 2,3-butanediol were produced from 100 g of sucrose at 37°C.

A. PRODUCTION FROM ACID-HYDROLYZED STARCHES.—Ward, Pettijohn, and Coghill[3] have studied the production of 2,3-butanediol from acid-hydrolyzed corn and wheat starches by *Aerobacter aerogenes* NRRL-B199 Research was carried out on the starches since it was believed that 2,3-butanediol could be recovered more readily from fermented mashes prepared from them than from fermented mashes prepared from whole grain.

Stock cultures of *A. aerogenes* were carried on slants of agar (containing 5 g. of Difco tryptone, 1 g. of glucose, 5 g. of Difco yeast extract, and 15 g of agar per liter).

The starter (inoculum) for the fermentation mashes was prepared by growing *A. aerogenes* in a special medium composed of the following ingredients to which was added subsequently separately sterilized 20 per cent urea solution:

Glucose	50 g
KH₂PO₄	0 60 g
MgSO₄ 7H₂O	0 25 g
CaCO₃	5.0 g.
Water, distilled, to make 1 liter	

The above medium was distributed into 200-ml. Erlenmeyer flasks in 100-ml. amounts and sterilized with steam at a pressure of about 15 lb. per sq. in. (121°C.) for 30 min. At the time of inoculation each flask received 1 ml. of sterile 20 per cent urea solution.

[1] SCHEFFER, M A , Thesis, Delft (1928).
[2] FULMER, E. I., L. M. CHRISTENSEN, and A R. KENDALL, *Ind. Eng. Chem.*, 25: 798 (1933)
[3] WARD, G. E., O G PETTIJOHN, and R. D. COGHILL, *Ind Eng. Chem.*, 37: 1189 (1945).

The fermentation mashes were prepared by cooking with steam, under pressure, suspensions of starch in water that had been acidulated with hydrochloric or sulphuric acids, and by adding the necessary salts and calcium carbonate. The concentration of starch used was generally about 10 per cent. Sufficient chemically pure acid was added to the starch slurries to yield an acid concentration of 0.02 to 0.12 normality. Before pressure-cooking the mashes, the starch slurries were gelatinized in hot-water baths while being agitated. They were then cooked for 1.25 to 4 hr. at a steam pressure of 22 to 25 lb. per sq. in. (128 to 131°C.), during which time the starches were saccharified. Table 98 shows the effect of cooking conditions on yields of 2,3-butanediol from starch 2778, a refined wheat starch containing 8.19 per cent moisture and 98 per cent starch on a dry basis.

TABLE 98.—EFFECT OF COOKING CONDITIONS ON BUTANEDIOL YIELD FROM STARCH 2778[1]

(Mashes 1 and 2 hydrolyzed 1.25 hr. at 130°C. mashes 3 and 4 hydrolyzed 4 hr. at 130°C., distilled water used throughout)

Fermenter No.	HCl concentration	Sugar, g./100 ml.			Fermentation time, hr.	Butanediol	Products, g./100 ml.	
		Original		Final			Acetoin	Ethyl alcohol
		Free	Total	Free				
1	0.12 N	10.01	10.36	0.30	32	3.72	0.20	0.33
2	0.12 N	10.35	10.36	0.38	31	3.74	0.11	0.21
3	0.01 N	10.25	10.30	0.52	*47	4.23	0.21	0.07
4	0.02 N	10.22	10.48	0.49	40	3.48	0.16	0.20

[1] WARD, G. E., O. G. PETTIJOHN and R. D. COGHILL, *Ind. Eng. Chem.* **27**, 1187 (1945).

Salts to supply the nutritive requirements of *A. aerogenes* were added to the starches in the following amounts per liter: 0.25 g. of $MgSO_4 \cdot 7H_2O$, 0.60 g. of KH_2PO_4, 2 g. of urea, and 5 to 10 g. of $CaCO_3$. The magnesium sulphate and potassium dihydrogen phosphate were added to the starch slurries before cooking. The urea (as a 20 per cent solution) and calcium carbonate (dry) were sterilized separately and added to the saccharified starches before inoculation. The pH was adjusted to 5.5 to 6.0 by means of the added $CaCO_3$.

The fermentations were carried out in rotary drums of the type described on page 609 under the following conditions: temperature = 30°C., revolutions per minute = 10 to 12, gauge pressure = 5 lb., air flow = 50 to 100 ml per min. They were concluded in each case when tests showed

that the minimum reducing sugar concentration was reached, which coincided with the maximum production of 2,3-butanediol.

The yields of 2,3-butanediol, acetoin, and ethyl alcohol obtained from acid hydrolyzates of starch 2778, as calculated in terms of pounds per 100 lb. of starch and per 34 lb. of pure dry starch (the equivalent of 1 bu. of corn), and of per cent of theoretical on the basis of the glucose consumed, are shown in Table 99.

TABLE 99.—YIELDS FROM STARCH 2778*

Fermenter No.	Yield, lb./100 lb. starch (as received)			Yield, lb./34 lb. pure dry starch			Yield, per cent of theoretical, based on glucose consumed[1]		
	Butane-diol	Ace-toin	Ethyl alcohol	Butane-diol	Ace-toin	Ethyl alcohol	Butane-diol	Ace-toin	Ethyl alcohol
1	32 5	1 8	2 9	12 3	0 7	1.1	75	4	7
2	32 7	1 0	2 2	12.4	0.4	9 8	74	2	5
3	36 8	1 9	0 6	13 9	0 7	0.2	86	4	1
4	36 4	1 4	1 8	13 8	0 5	0 7	85	3	4

* WARD G L., O. G. PETTIJOHN, and R. COGHILL, *Ind Eng. Chem*, **37**: 1189 (1945).
[1] "Glucose consumed" is assumed to equal original free sugar minus final free sugar.

Although good yields of 2,3-butanediol were obtained from starch 2778, considerably poorer yields were obtained from another wheat starch, designated as SS, which was hydrolyzed and fermented in a similar manner. This observation led Ward and his associates to investigate the effect of growth factors; of extraction of starches with an acid, alkali, and alcohol; of added ions; and of ion-exchange agents. The effect of some of these factors on yields will be discussed briefly.

The addition of growth factors—biotin, inositol, nicotinic acid, p-aminobenzoic acid, pyridoxin, riboflavin, sodium pantothenate, and thiamin—to aerated glucose cultures did not appear to produce any differences in the proportions of 2,3-butanediol and ethyl alcohol.

Portions of starch SS extracted in the cold with 0.1 N HCl, 91 N NaOH, and 65 per cent aqueous ethyl alcohol were used in the customary manner for the preparation of mashes. The yield of 2,3-butanediol was increased to 72 per cent of the theoretical, but the yield of ethyl alcohol was greatly decreased as a result of extraction with 0.1 N NaOH. Moreover, no improvement in yields resulted from the use of portions of starch extracted with HCl or ethyl alcohol.

As the result of finding marked differences in the contents of trace

elements in the ashes of starches 2778 and SS after spectroscopic analyses, Ward and his coworkers investigated the effect of added ions. The addition of copper ions in concentrations of 0.01, 0.10, and 1.0 part per million to a medium prepared from hydrolyzed starch SS, greatly improved the yield of 2,3-butanediol but reduced the yield of ethyl alcohol. The addition of 0.01 p p m of copper (as CuSO₄) to the hydrolyzates of 24 corn and wheat starches led to variable results.

The addition of manganese, cobalt, and molybdenum ions to acid-hydrolyzed starches resulted in lowered yields of 2,3-butanediol and increased yields of ethyl alcohol under aerobic conditions, and a faster rate of fermentation under anaerobic conditions.

Ion-exchange agents, such as Zeo-Karb II, were found to be particularly desirable for the treatment of starch hydrolyzates. They were effective in correcting unbalances in the ions and in removing excessive quantities of iron and copper picked up from the equipment used for the preparation of the hydrolyzates.

In the optimum use of ion-exchange agents, such as Zeo-Karb II, the acid hydrolyzate was adjusted to a pH of 5 1 and filtered to remove the small amount of flocculent precipitate present. One liter of the filtrate was agitated for 30 min. with 20 g of Zeo-Karb II. The ion-exchange agent was then removed by filtration, the nutrient salts were added, and the mash was sterilized, cooled, and then inoculated. An alternate and more efficient method for treating the acid-hydrolyzate with the ion-exchange agent was to pass the former continuously through a column containing the latter.

It may be concluded from the researches of Ward and his coworkers that hydrolyzed starch mashes properly balanced in respect to ions, perhaps by the use of an ion-exchange agent, and supplied with the usual nutrient materials, may be fermented under optimum conditions by *A. aerogenes* with the production of satisfactory yields of 2,3-butanediol However, higher yields on the average may be obtained from acid-hydrolyzed whole grain mashes which do not require the same degree of adjustment

B PRODUCTION FROM WOOD HYDROLYZATES —Perlman has investigated the production of 2,3-butanediol from acid hydrolyzates of hard and soft woods, including southern red oak, Douglas fir white spruce, and southern yellow pine A summary of his researches follows.

A culture of *A. aerogenes* NRRL 199 was employed This was grown in a special medium containing 5 0 per cent of glucose 0 5 per cent of corn steep liquor, and 0.5 per cent of CaCO₃, which was aerated continuously on a mechanical shaker for 12 to 16 hr before it was used The

' Perlman, D , Ind Eng Chem, 36: 803 1944

hydrolyzates as is shown in Table 101, on page 495. He discovered also that the neutralization method of treating wood hydrolyzates was satisfactory when the organism had been acclimatized.

C. PRODUCTION FROM GLUCOSE.—Olson and Johnson[1] described the production of 2,3-butanediol from glucose. Under optimum condition wherein aeration was used, yields of 80 millimoles of 2,3-butanediol were obtained per 100 millimoles of glucose with *Aerobacter aerogenes* 199 Sugar in concentrations up to 10 per cent was fermented in less than 48 hr Sugar in concentrations up to 26.5 per cent was fermented in 108 hr. when it was fed slowly as a concentrated substrate during the course of the fermentation. In fermentations such as the latter, the concentration of glycol obtained was as high as 98 g. per liter.

2. *The Bacillus polymyxa Fermentation.*—This fermentation is characterized by the production of the levorotatory form of 2,3-butanediol and ethanol from mashes containing saccharified ground whole grains, such as wheat.

Some of the first contributions concerning the fermentation were furnished by Donker,[2] who studied carbohydrate dissimilation by *Aerobacillus polymyxa* (Prazmowski) Donker. Later Kluyver and Scheffer[3] investigated the fermentation. During the years 1942-1946 a very large amount of intensive research was carried out on this fermentation, particularly by Ledingham, Adams, Stanier, and their associates of the Canadian National Research Laboratories, and Katznelson and Lochhead of the Canadian Department of Agriculture.

The fermentation may be conveniently discussed under the following headings. the organism, the inoculum, and other factors affecting the fermentation.

THE ORGANISM.—The fermentation is carried on by *Bacillus polymyxa* (Prazmowski) Migula There are other synonyms for this species, such as *Aerobacillus polymyxa* (Prazmowski) Donker, *Clostridium polymyxa* (Prazmowski), *Granulobacter polymyxa* (Beijerinck), and *B asterosporus* (A. Meyer) Migula.

The organism has been described by Donker,[4] by Porter, McCleskey, and Levine,[5] by Ledingham, Adams, and Stanier,[6] and by others.

Bacillus polymyxa is a sporeforming, Gram-negative, facultative, aerobic organism of the *Aerobacillus* Donker group and family *Bacillaceae*

[1] OLSON, B. H., and M. J. JOHNSON, *Jour. Bact.*, 55 (No. 2): 209 (1948)
[2] DONKER, H. J L., Thesis, Delft, 1926.
[3] KLUYVER, A J., and M. A. SCHEFFER, U S. Patent 1,899,156, Feb. 28, 1933
[4] DONKER, *op. cit.*
[5] PORTER, R., C S McCLESKEY, and M LEVINE, *Jour. Bact*, 33: 163 (1937)
[6] LEDINGHAM, ADAMS, and STANIER, *loc. cit.*

It is motile with peritrichous flagella and occurs vegetatively as rods that vary in size from about 0.5 to 1 micron in width to 2 to 8 microns in length. It liquefies gelatin, produces catalase, hydrolyzes starches, and gives a positive Voges-Proskauer reaction. Most strains ferment arabinose, cellobiose, dextrins, galactose, glucose, inulin, lactose, levulose, mannitol, mannose, raffinose, salicin, sorbitol, starch, trehalose, and xylose. Some, but not all, strains ferment rhamnose. The optimum temperature appears to be about 30°C.

Strains of *B. polymyxa* may be isolated from a wide number of sources including soil, water, milk, grains, feces, and fresh or decaying plants.

Ledingham, Adams, and Stanier[1] recommended the following procedure for its isolation: Agitate some soil or other source material with sterile water. Place 5 ml. of the suspension thus prepared in a sterile culture tube and pasteurize it in a water bath at a temperature of 80°C for 10 min. Inoculate tubes containing sterile lactose or starch broth with 1-ml. portions of the pasteurized suspension and incubate the tubes at 30°C. Streak plates containing neutral red, starch-peptone, or starch-yeast agar with material from the tubes showing gas production after 2 to 3 days. Select and isolate typical colonies.

The colonies of *B. polymyxa*, which show a wide range of variability according to Ledingham and his associates, are generally pink or red in color when grown on the neutral red agar. They give rise to a pleasant, fruit-like odor. Some, which form a thick slime, may become distended by gas bubbles. Growth and spreading of the colonies on moist plates occur rapidly.

TABLE 102 THE COMPOSITION OF SOME MEDIA USED IN ISOLATING *B. polymyxa*[1]

Starch broth, per cent		Neutral red agar	
Peptone	1	Starch or lactose	20 g
Starch	1.5	Peptone	10 g
		Yeast extract	5 g
Lactose broth, per cent		Neutral red	0.05 g
Peptone	1	Agar	15 g
Lactose	1	Water	1000 ml

Data obtained from article by G. A. Ledingham, G. A. Adams and R. A. Stanier, *Can. Jour. Research F*, 23: 48 (1945).

Strains of *B. polymyxa* should be selected primarily for their ability to produce high yields of 2,3-butanediol and ethanol, and also on the basis of the filterability of the mashes fermented by them. Stability of charac-

Ledingham, Adams, and Stanier, loc. cit.

teristics is another factor of importance in the selection of the most suitable strains for fermentation.

Katznelson[1] observed that there was a difference in the filterability of mashes, some filtering within a few minutes and others requiring hours. The difference in filterability was found to be a strain characteristic.

Stanier, Adams, and Ledingham[2] studied strains of *B. polymyxa* in relation to the production of 2,3-butanediol and the filterability of the fermented mashes. They found that it is impossible to correlate colony appearance with filterability and yields. Filtration of mashes fermented by certain strains, including both high and low producers of 2,3-butanediol, was readily accomplished while that of other strains was accomplished with great difficulty. Mashes were classified on the basis of their filterability. A Type A mash was one in which the residual solids of fermentation collected on the surface of the medium, leaving a rather clear liquid below. The Type B mash was one in which the residual solids accumulated as a loose sediment with a relatively clear layer of liquid above. The Type C mash was one in which the solids were held in a fairly uniform suspension as a result of the large amount of loose slime produced by the organism. Both Type A and Type B mashes presented no difficulties in respect to filtration, but the Type C mash filtered very slowly. Strains producing the Type C mash, even though good producers of butanediol, are not considered to be practical for commercial use.

Stanier and his associates[3] pointed out the value of preserving strains of *B. polymyxa* by drying in order to prevent further variations.

THE INOCULUM.—The inoculum is the starter used to seed the main fermentation medium. Particular attention must be paid to the selection of an inoculation medium that will favor the rapid development of the bacteria and produce optimum yields of the desired end products. With respect to the use of the inoculum, it is necessary to know at what age it should be employed and in what quantity.

Ledingham, Adams, and Stanier[4] used two types of inoculation media, which are listed below. Medium 1 has the advantage that it may be pipetted more readily than Medium 2.

Medium 1	Medium 2
Soluble starch, 2%	Whole wheat, 5%
Yeast extract, 0 5%	Yeast extract, 0 5-1.0%
Calcium carbonate, 1%	Calcium carbonate, 1%

[1] Katznelson, H., *Can. Jour. Res*, **C, 22**: 235 (1944).

[2] Stanier, R. Y., G. A. Adams, and G. A. Ledingham, *Can. Jour. Research*, F, **23**: 72 (1945).

[3] *Ibid*

[4] Ledingham, Adams, and Stanier, *loc. cit*

The yeast extract used in these media may be substituted by malt sprouts, corn steep liquor, malt extract, or dried yeast.

Katznelson used, in addition to the above media, the Difco Methyl Red—Voges-Proskauer Medium.

The age of the inoculum, within the limits of 10 to 96 hr., apparently makes little difference on the yield of 2,3-butanediol and ethyl alcohol provided that the organism is active.

The size of the inoculum used (provided that it represents at least 1 per cent of the volume of the fermentation medium) is also not too influential on the yields. Katznelson found that an inoculum of 0.5 per cent size was too small, but that the use of quantities representing 2.5 to 5.0 per cent of the volume of the fermentation mash was satisfactory. Larger amounts may be used, of course, if desired.

Table 103 shows the influence of age of inoculum, quantity of inoculum, and nature of the inoculating medium on yields of 2,3-butanediol by a strain of *B. polymyxa*.

TABLE 103—INFLUENCE OF AGE AND QUANTITY OF INOCULUM AND INOCULATING MEDIUM ON YIELD[1] OF BUTANEDIOL BY STRAIN 47

Age of inoculum, hr	2,3-Butanediol, per cent	Amount of inoculum, per cent	2,3-Butanediol, per cent	Nature of inoculating medium	2,3-Butanediol, per cent
24	3.16	0.5	2.60	Yeast-starch-$CaCO_3$	3.09
48	3.21	2.5	3.04	Difco M R—V.P	3.13
96	3.17	5.0	3.04	5% whole-wheat mash	3.08

[1] KATZNELSON, H., Can. Jour. Research, C, 22: 235 (1944).

RAW MATERIALS USED.—*B. polymyxa* is capable of fermenting a variety of properly prepared grain mashes, such as corn and wheat; glucose; xylose;[1] mannitol,[1] and other carbohydrates. The utilization of corn or whole wheat appears desirable when these grains are available in large quantities. The use of whole wheat has been studied extensively by Ledingham and his associates and by Katznelson.

CONCENTRATION OF RAW MATERIALS.—The choice of the optimum concentration of raw material must be based on fermentation efficiency and mash viscosity. Ledingham and his coworkers[2] found that mashes which contained more than 15 per cent of wheat by weight were inefficiently fermented and were generally thick and viscous. Such mashes gave higher yields than those of lower concentrations, but the time required for fermentation was prolonged and the starches were incompletely

[1] ADAMS, G. A., and R. Y. STANIER, Can. Jour. Research, B, 23: 1 (1945).
[2] LEDINGHAM, ADAMS, and STANIER, loc. cit.

utilized. A concentration of whole wheat of approximately 15 per cent was considered best. Viscosity is a factor of importance especially in connection with handling or pumping of the mashes.

EFFECT OF PARTICLE SIZE OF WHEAT.—The investigators at the Canadian Research Laboratories found that the yields of 2,3-butanediol and ethyl alcohol were not adversely affected by the particle size of wheat provided that the kernels were broken.

PREPARATION OF THE MASH.—The preparation of the mash includes the weighing out and the mixing of the ingredients as well as its cooking or sterilization.

Ledingham and his collaborators placed 300-ml. portions of medium, after preliminary treatment, into 500-ml. Erlenmeyer flasks. A 15 per cent mash thus contained 45 g. of wheat in 300 ml. of medium. To each mash, 1 per cent of calcium carbonate was added, except when special tests were carried out to determine the effect of pH or carbonates In order to avoid the caking of starches in the mashes during sterilization, the ingredients were gelatinized by cooking lightly with constant agitation and then placed in the Erlenmeyer flasks. Sterilization was ordinarily accomplished by the use of steam at a pressure of 15 lb. (121°C) for 1 hr , a relationship established after experiments had been carried out to determine the effect of various combinations of temperature and time of cooking on the production of 2,3-butanediol and ethanol. It was discovered that cooking mashes for 3 hr. at temperatures of 126°C. or higher resulted in lowered yields. Table 101 indicates that the time and temperature of cooking may be varied considerably without detrimental effects on the yields.

Ledingham and his associates occasionally preliquefied their grain mashes by the addition of malt (1 per cent of the weight of the grain) and by holding the temperature at 70°C. for 10 min. This facilitated the subsequent agitation of the mash and the uniform dispersion of the inoculum.

Katznelson prepared mashes of the following composition in 1-liter Erlenmeyer flasks: 17 8 g of coarse whole wheat flour, 1 g. of calcium carbonate, and 100 ml. of water. The concentration of wheat was thus 15 per cent, based on a total mash weight of 118 8 g. The mashes were cooked for 1 hr. with steam at a pressure of 15 lb. per sq in. Katznelson reported that the ratio of surface area to volume produced higher yields of 2,3-butanediol and ethyl alcohol produced under these circumstances than were obtained when larger amounts of mash were used in a flask of the same size.

pH CONTROL.—The optimum pH for the production of 2,3-butanediol and ethyl alcohol from whole wheat mashes of approximately 15 per cent

TABLE 101 —EFFECT OF COOKING TIME AND TEMPERATURE ON 2,3-BUTANEDIOL AND
ETHANOL PRODUCTION BY *Bacillus polymyxa* C 3(2) AFTER 72-HR FERMENTATION
ON 15 PER CENT MASH[1]

Time of cooking, hr	Temperature, °C					
	100	108 1	115 1	121 0	126 0	130 4
	2,3-Butanediol, per cent					
½	2 58	2 77	2 75	2 67	2 62	2 67
1	2 72	2 77	2 65	2 81	2 70	2 73
3	2 95	2 81	2 57	2 67	2 15	1 65
	Ethanol, per cent					
½	1 71	1 74	1 72	1 63	1 67	1 76
1	1 77	1 77	1 69	1 61	1 63	1 55
3	1 86	1 69	1 61	1 47	1 14	0 78

[1] LEDINGHAM G A, G A Adams and R Y Stanier, *Can Jour Research* F, 23: 48 (1945)

concentration by *B polymyxa*, appears to be within the range of 5.6 to 6.5.

The pH of unbuffered wheat mashes lies close to neutrality (6.8 to
7.0), according to Ledingham and his associates,[1] but drops to 5.5 to 5.0
during the fermentation unless a neutralizing agent is added to the
medium

Although the pH of the fermentation mash may be controlled by
several different agents, the use of calcium carbonate or ammonia appears
to produce particularly favorable results   In the laboratory, the use of 1
per cent calcium carbonate prevents the pH from falling below 5 6 to 5 8.

Other carbonates, such as barium and magnesium, are not as effective
as calcium carbonate when employed at the same concentration, according
to Ledingham, Adams, and Stanier.[1] The use of magnesium carbonate
results in a reduction of the butanediol ethanol ratio

Adams and Leslie[2] reported that the use of calcium carbonate on a
commercial scale may offer certain disadvantages For example, they
stated that the pH cannot be maintained at a uniform level throughout
the fermentation period, that cooking mashes which contain calcium
carbonate has an adverse effect on the subsequent fermentation, and that
the presence of large amounts of calcium carbonate in the unfermented
residues lowers their feed value for animals on account of the larger ash
contents

[1] LEDINGHAM, Adams, and Stanier, *loc cit*
[2] Adams, G A, and J D Leslie, *Can Jour Research* F, 24: 12 (1946)

Adams and Leslie,[1] who studied pH control in relation to the *B. polymyxa* fermentation, advocated the use of ammonia because it eliminated the disadvantages resulting from the use of calcium carbonate, because it is abundant and relatively inexpensive, because it is usually free from microorganisms, and because it may be handled conveniently. They found that the maintenance of a pH range of 5.8 to 6.0 with ammonia was optimum. It was favorable for the production of 2,3-butanediol and ethanol; it minimized acid production and decreased the amount of ammonia required. The use of ammonia did not affect adversely the butanediol/ethanol ratio, which was about 1.5.

TEMPERATURE.—The temperatures most favorable for the production of 2,3-butanediol and ethanol by *B. polymyxa* appear to lie close to 30°C Ledingham, Adams, and Stanier[2] suggested that a temperature of 32.5°C might be most satisfactory, while Katznelson incubated his mashes at 28°C. A temperature of 30°C has been used most frequently in the investigations carried out by the Canadian National Research Laboratories.

SURFACE-VOLUME RATIO.—The effect of the ratio of surface area to volume of fermentation mashes was first investigated by Katznelson,[3] who found that the yields of 2,3-butanediol and ethyl alcohol were decreased as the quantities of mash in 4-liter Erlenmeyer flasks were increased from 500 ml. to 2,000 ml. by increments of 500 ml. The ratio of butanediol/ethanol was also found to decrease as the volume of mash increased. The highest yields and shortest fermentation periods occurred when the flasks contained shallow layers of mash

Adams and Leslie[4] have confirmed the observations made by Katznelson and made other significant contributions concerning the effects of the surface-volume ratio and reduced pressure on the fermentations by *B. polymyxa* and *A. aerogenes*. As a result of many experiments, they found that the yields of 2,3-butanediol and ethanol were increased in media which were fermented in shallow layers, both under aerobic and anaerobic conditions. However, under anaerobic conditions (produced by the use of nitrogen gas), the yields of ethanol were greater and those of 2,3-butanediol were smaller than those obtained from controls fermented in the presence of air.

The results obtained by Adams and Leslie in determining the effect of aerobic and anaerobic conditions are shown in the following table In carrying out the tests that led to the results tabulated, 300-ml. portions of

[1] ADAMS and LESLIE, *loc. cit*

[2] LEDINGHAM, ADAMS, and STANIER, *loc. cit.*

[3] KATZNELSON, *loc. cit*

[4] ADAMS and LESLIE, *op. cit.*, p. 107.

wheat mash, of 15 per cent concentration and containing 1 per cent of calcium carbonate, were placed in 500-, 2,000- and 6,000-ml. Erlenmeyer flasks, sterilized, inoculated with 3 per cent of inoculum (a 5 per cent whole wheat–yeast–water–calcium carbonate medium), and incubated at 30°C. Nitrogen was passed over the surface of the mashes in one series of experiments while the mashes of the control series were allowed to ferment in the presence of the gases evolved.

TABLE 105.—EFFECT OF FERMENTING 300 ML. OF WHOLE-WHEAT MASH WITH B. polymyxa UNDER AEROBIC AND ANAEROBIC CONDITIONS AT VARIOUS SURFACE-VOLUME RATIOS[1]

Fermentation period, hr	Surface, cm², volume, ml	Butanediol, per cent		Ethanol, per cent		Total products per cent		Ratio	
		Air	Nitrogen	Air	Nitrogen	Air	Nitrogen	Air	Nitrogen
21	0.16	0.78	0.93	0.41	0.53	1.19	1.46	1.90	1.75
	0.66	1.53	1.36	0.65	0.83	2.18	2.19	2.35	1.62
	1.32	1.96	1.62	0.76	1.07	2.72	2.69	2.58	1.31
48	0.16	1.96	1.62	0.84	1.26	2.80	2.88	2.33	1.23
	0.66	2.75	2.54	1.38	1.77	4.13	4.31	2.00	1.44
	1.32	2.93	2.66	1.33	1.76	4.26	4.42	2.20	1.51
72	0.16	2.22	2.03	1.17	1.26	3.39	3.29	1.90	1.61
	0.66	2.93	2.54	1.39	1.77	4.32	4.31	2.11	1.44
	1.32	3.06	2.66	1.18	1.76	4.24	4.42	3.06	1.51

[1] Adams, G. A. and J. D. Leslie. *Can. Jour. Research*, F, 24, 197 (1946).

Other experiments carried out by Adams and Leslie indicated that mashes under an atmosphere of carbon dioxide fermented at about the same rate regardless of the depth of the medium. The fact that fermentation was inhibited under an atmosphere of carbon dioxide in mashes with a large surface volume ratio, suggested that rapid fermentation of mashes in thin layers was due to the escape of carbon dioxide.

EFFECTS OF REDUCED PRESSURE.—Experiments were also carried out by Adams and Leslie to determine the effect of reduced pressure on the rate of fermentation and yield of products by B. polymyxa from 15 per cent whole wheat mashes. Typical results are shown in Table 106. In general the effect of the use of low pressure was to reduce the fermentation time and the butanediol ethanol ratios. Fermentations under reduced pressure were about 95 per cent complete in 18 hr., whereas those at atmospheric pressure were only about 60 per cent complete. Ethanol

production increased at the expense of 2,3-butanediol production; however, the combined yields of the two products under reduced pressure approached the theoretical yield of 4.6 per cent. It is noteworthy also that the pH dropped to a low of 5.78 in 24 hr. and then increased until the

TABLE 106 —EFFECT OF REDUCED PRESSURE ON RATE OF FERMENTATION AND
PRODUCT YIELD BY *B. polymyxa*[1]

Fermentation time, hr	Pressure, in. of Hg	Butanediol, per cent	Ethanol, per cent	Total products, per cent	Ratio	pH
24	30	0.85	0.51	1 36	1.67	6 17
	10	1 60	1.14	2.80	1 45	5 73
48	30	1 76	1 03	2.79	1.71	6 00
	10	2 44	1.92	4.36	1.27	5 80
72	30	2 47	1.52	3.99	1.62	5 95
	10	2 51	1 90	4 47	1 28	5 95
96	30	2 65	1.73	4.38	1.53	5.90
	10	2 52	2.02	4.45	1.25	5.97
120	30	2 76	1.78	4.54	1 55	5 85
	10	2 52	2 06	4 58	1.23	5 98

[1] ADAMS, O. A., and J D LESLIE, *Can Jour Research*, F, 24: 107 (1946).

end of the fermentation, a fact that Adams and Leslie suggested might be due to the proteolytic breakdown of wheat proteins to yield ammonia

AEROBIC AND ANAEROBIC FERMENTATIONS.—The effect of passing various gases through the fermentation medium was studied by Adams[1] Aeration was accomplished by passing 333 ml of gas per minute per liter of mash through the medium. The gases used were air, oxygen, nitrogen, hydrogen, and carbon dioxide.

Under aerobic conditions, which were obtained by the passage of air or oxygen through the media, the yields of 2,3-butanediol were increased, but those of ethanol were decreased in comparison with the controls which were neither aerated nor oxygenated. Oxygenation and aeration increased the butanediol/ethanol ratio and the rate of the formation of butanediol, but it did not shorten the fermentation period

Under anaerobic conditions, which were produced by the passage of nitrogen or hydrogen gases through the media, the results were somewhat different. Both nitrogen and hydrogen gases increased the rate of formation and final yield of ethanol.

The results of the fermentation of 15 per cent whole wheat mashes under aerobic and anaerobic conditions are shown in Table 107. It may be observed from an examination of the table that the use of carbon

[1] ADAMS, G A., *Can Jour Research*, F, 24: 1 (1946).

dioxide resulted in yields of butanediol and ethanol which were similar to those obtained when nitrogen and hydrogen gases were used and that the fermentations were largely completed in 72 hr.

EFFECT OF AGITATION.—Adams[1] investigated the effect of continuous and intermittent agitation by mechanical shakers on the production of 2,3-butanediol and ethanol by *B. polymyxa*. He found that continuous agitation inhibited the production of 2,3-butanediol and ethanol (particularly the ethanol) and increased the butanediol/ethanol ratio. Results with intermittent agitation were similar.

TABLE 107—FERMENTATION OF 15 PER CENT WHOLE-WHEAT MASH UNDER AEROBIC AND ANAEROBIC CONDITIONS BY *B. polymyxa*[1]

Organism	Aeration treatment	Butanediol, per cent					Ethanol, per cent				
		Time, hr					Time, hr				
		21	48	72	96	120	21	48	72	96	120
CB (2)	Control	0.76	1.85	2.41	2.60	2.70	0.48	1.13	1.55	1.65	1.60
	Air	0.86	1.91	2.68	2.91	2.88	0.44	0.86	1.23	1.33	1.31
	O₂	1.13	2.73	3.06	3.01	3.01	0.42	0.95	1.08	1.10	1.09
	N₂	1.31	2.33	2.44	2.47	2.48	0.86	1.82	1.88	1.87	1.85
	CO₂	1.65	1.93	2.40	2.52	2.59	0.45	1.23	1.73	1.90	1.84
	H₂	0.93	1.99	2.43	2.59	2.62	0.65	1.71	1.86	1.91	1.91
CI (2)	Control	0.67	1.47	2.18	2.51	2.71	0.40	0.81	1.23	1.51	1.65
	Air	0.61	1.36	2.28	2.90	3.00	0.30	0.55	0.66	1.05	0.98
	O₂	1.02	2.03	2.88	2.97	3.05	0.36	0.56	0.80	0.87	0.91
	N₂	1.10	2.47	2.51	2.49	2.51	0.73	1.76	1.88	1.85	1.83
	CO₂	0.70	1.62	2.35	2.46	2.51	0.43	1.00	1.46	1.62	1.61
	H₂	0.91	2.13	2.51	2.51	2.51	0.56	1.44	1.73	1.90	1.90

[1] Adams G. A. *Can. Jour. Research F*, 26, 1 (1948)

EFFECT OF YEAST EXTRACT AND WHEAT FRACTIONS.—The addition of yeast extract to a whole-wheat mash may stimulate the production of 2,3-butanediol, according to Katznelson.[3] The largest degree of stimulation was produced by 1 g of yeast extract per 15 g of whole wheat in a 15 per cent mash. The ashing of the yeast extract destroyed its stimulating properties. Ledingham, Adams, and Stanier[4] demonstrated (refer to Table 108) that yeast extract in 0.25 per cent concentration increased the yield of butanediol and ethanol from whole-wheat flour and other wheat

[1] *Pr cl*
[3] KATZNELSON, *loc. cit.*
[4] LEDINGHAM, ADAMS, and STANIER, *loc. cit.*

fractions. However, it was found that a concentration of greater than 1 per cent yeast extract could decrease the rate of fermentation. The use of 3 per cent corn-steep liquor produced results similar to those obtained with the use of 0.25 to 0.5 per cent yeast extract.

TABLE 108 —EFFECT OF WHEAT FRACTIONS AND YEAST EXTRACT ON FERMENTATION[1]

	2,3-Butanediol + ethanol, per cent			
	Strain 233b (2)		Strain 51 CR	
Wheat fraction	Yeast extract added			
	None	0 25 per cent	None	0 25 per cent
Whole-wheat flour	3 33	3.56	3 17	3 54
Starch + wash water	2 49	3 00	2.56	3 03
Starch + wash water + bran	2 90	3 26	3.00	3 30
Whole-wheat flour .	2 78	3 20	3 12	3 26
Starch + wash water + bran	2 68	3 00	2 93	3 00
Starch + wash water + bran + half the gluten	2 71	2 90	2 82	3 00
Starch + wash water + bran + all the gluten	2 78	3 01	2 80	2 99

[1] LEDINGHAM, G. A., G A ADAMS, and R. Y STANIER, Can. Jour Res, F, 23:45 (1945)

As the result of research to determine which constituents of whole wheat were essential for the fermentation medium, it was ascertained by Ledingham and his associates that the bran, shorts, germ, and soluble nitrogen constituents were essential for a normal fermentation. However, it was found that the removal of gluten had no particular effect on the results

EFFECT OF GROWTH FACTORS ON THE FERMENTATION OF STARCH — Although wheat starch with inorganic supplements is only partially utilized by *B. polymyxa*, Fratkin and Adams[1] found that 8 per cent starch mashes could be fermented in a satisfactory manner after the addition of suitable organic nutrients. For example, a starch–whole-wheat–wash-water mash supplemented with 1 per cent of malt sprouts or 2.5 per cent of shorts appeared to yield best results. The table reproduced below summarizes some of the findings of Fratkin and Adams with respect to the use of various nutrients, such as malt sprouts, shorts, bran, cerogras, alfalfa, soya beans, yeast extract, and corn-steep liquor, in different concentrations, in starch–whole-wheat–wash-water mash fermentations.

[1] FRATKIN, S B , and G. A ADAMS, *Can. Jour Research*, F, 24: 29 (1946).

TABLE 109 —EFFECT OF VARIOUS SUPPLEMENTS IN VARYING CONCENTRATIONS IN A STARCH–WHOLE-WHEAT–WASH-WATER MASH[1] FERMENTATION[2]

Nutrient	Per cent	2,3-Butanediol + ethanol, per cent		
		48 hr.	72 hr.	96 hr.
Control		1 61	2 90	3 52
Malt sprouts[3]				
Sterilized	1 0	2 95	4 03	4 21
Unsterilized	0 5	3 43	0 76*	0 80*
Shorts[4]	1 0	2 01	3 64	4 11
Bran[5]	1 0	2 19	3 57	4 16
Cerograss[6]	1 0	2 61	3 61	4 03
Alfalfa	1 0	2 49	3 87	4 06
Soya beans	1 0	1 48	3 13	3 80
Yeast extract	0 5	2 72	3 75	3 89
Corn-steep liquor	0 5	2 09	3 13	3 51
Malt sprouts				
Sterilized	2 0	3 14	4 25	4 45
Unsterilized	1 0	3 61	4 24	4 11*
Shorts	2 0	1 95	3 80	4 23
Bran	2 0	2 03	3 68	4 22
Cerograss	2 0	2 85	3 85	4 18
Alfalfa	2 0	2 91	3 77	4 01
Soya beans	2 0	2 03	3 58	4 12
Yeast extract	1 0	2 88	3 75	3 96
Corn-steep liquor	1 0	2 47	3 51	3 80
Malt sprouts				
Sterilized	3 0	3 44	4 36	4 48
Unsterilized	1 5	3 37	2 40*	2 30*
Shorts	3 0	2 21	4 11	4 38
Bran	3 0	2 05	3 54	4 22
Cerograss	3 0	2 90	3 87	4 17
Alfalfa	3 0	3 17	4 15	4 21
Soya beans	3 0	1 90	3 64	4 06
Yeast extract	1 5	2 87	3 58	3 95
Corn-steep liquor	1 5	1 93	3 16	3 57

[1] Starch content of mash 7 93 per cent.
[2] Inoculum S. B. and O. A. Anacne Can. Jour. Research F, 24, 29 (1946).
[3] Fermentable log in malt approx to 22 50 per cent.
[4] Starch content of shorts 41 35 per cent.
[5] Starch content of bran 7 88 per cent.
[6] Cerograss dehydrated young oat plants of tunnel from Grosvenor & Co. Ltd. Wallaceburg Ont.
* Contaminated.

PRODUCTION FROM CORNSTARCH.—Kooi, Fulmer, and Underkofler (1948) reported on a procedure for the successful production of 2,3-butanediol from mashes containing cornstarch and nutrients by *Aerobacillus polymyxa*. The best medium contained 7.5 g. of commercial cornstarch, 0.5 g. of corn gluten, 0.006 g. of potassium permanganate, and 0.5 g. of calcium carbonate per 100 ml. The yields from this medium (26.8 per cent 2,3-butanediol, 1.1 per cent acetoin, and 14.8 per cent ethanol) were comparable to those obtained from corn mashes of an equivalent starch content.

It was found that increased yields of 2,3-butanediol were obtained from cornstarch and corn mashes when 0.5 per cent of dried brewers' yeast or malt sprouts was added to the corn-mash inoculum medium.

EFFECT OF GROWTH FACTORS AND NITROGEN SOURCES ON THE FERMENTATION OF A GLUCOSE-INORGANIC SALT MEDIUM.—The nutritional requirements of *B. polymyxa* have been studied by Katznelson and Lochhead[1] The basal inorganic medium used by them contained 1.0 g. of K_2HPO_4, 1.0 g. of KH_2PO_4, 0.2 g. of $MgSO_4 \cdot 7H_2O$, 0.1 g. of $NaCl$. 0.1 g. of $CaCl_2$, 0.01 g. of $FeSO_4 \cdot 7H_2O$, 0.01 g. of $MnSO_4 \cdot 4H_2O$, and 0.01 g of $ZnSO_4$ in 1,000 ml of distilled water. The salt solution was filtered after being heated to boiling and 5.0 g. of c.p. glucose and 2 g. of vitamin-free casein hydrolyzate were then added. The pH was adjusted to 6.8 The resultant medium was distributed into thoroughly cleaned and rinsed culture tubes and sterilized for 15 min. at a steam pressure of 15 lb. per sq in.

Studies concerned with growth factor requirements revealed that 82 strains of *B. polymyxa* would grow in the medium described above only when biotin was present, which established the essentiality of this vitamin The growth of some strains was stimulated by thiamin, whereas that of others was inhibited. Inositol, nicotinic acid, pantothenic acid, pyridoxine, and riboflavin appeared to be without effect on the growth of strains of *B. polymyxa*. Yeast extract, in a concentration of 3 g per liter, produced better growth in the medium containing only the inorganic salts and glucose than all of the other growth factors examined.

The nitrogen requirements of strains of *B. polymyxa* were also investigated by Katznelson and Lochhead.[2] Although hydrolyzed casein could be replaced by ammonium sulphate, urea, and a mixture of 18 amino acids, these substances were in general inferior to casein hydrolyzate Asparagine and potassium nitrate were found to be poor sources of nitrogen. Peptone and tryptone were about as effective as the casein hydrolyzate as sources of nitrogen. Yeast extract produced the most

[1] KATZNELSON, H., and A. G. LOCHHEAD, *Can. Jour. Research, C*, **22:** 273 (1944)

[2] *Ibid.*

abundant growth, part of which may be attributed to growth factors it contained.

EFFECT OF BACTERIOPHAGE.—The effect of bacteriophage on strains of *B. polymyxa*, particularly in relation to the production of 2,3-butanediol, has been studied by Katznelson.[1] Freshly isolated bacteriophages and an old mixed bacteriophage which could be separated into eight distinct groups based on differences in size and internal structure of the plaques, were used in the investigation, as well as 82 strains of *B. polymyxa* (44 of them freshly isolated).

When the 82 strains of *B. polymyxa* were tested with a mixture of all of the different types of bacteriophage, complete lysis of 63 strains and partial lysis of six strains took place. There appeared to be no relationship between the ability to produce 2,3-butanediol and susceptibility to bacteriophage in the cases of the strains of *B. polymyxa* examined.

The effect of temperature on bacteriophage was studied by Katznelson,[2] who found that a temperature of 55°C, for 30 min. inactivated it. However, it was ascertained that bacteriophage may be transmitted through bacterial spores and in one case a temperature of 80°C, for 60 min failed to destroy it Temperatures sufficiently high to destroy the bacteriophage also destroyed the spores of the strain of *B polymyxa* tested

In order to avoid the serious problems associated with bacteriophage that might arise in a production plant, Katznelson advocated the adequate sterilization of equipment and materials, the employment of aseptic techniques, and the use of cultures shown by careful examination to be free of bacteriophage and nonlysogenic He also suggested that strains of *B polymyxa* that formed spores be used and that the inoculum be pasteurized As a further precautionary measure, he suggested the use of an inoculum containing a combination of three resistant strains of *B polymyxa* when bacteriophage was known to be present Although mixtures of two or three highly resistant strains have produced good results, a single susceptible strain has usually produced better yields of 2,3-butanediol The combination of five or more strains (for example, of two susceptible and three resistant strains) has not produced a good inoculum

PILOT-PLANT PRODUCTION UNIT.—A small-scale process for producing 2,3-butanediol has been described by Rose and King[3] Their production unit has a capacity for about 25 gal of whole wheat mash per week

BIOCHEMISTRY OF THE FERMENTATION.—Studies concerning the biochemistry of the fermentation brought about by strains of *B polymyxa*

have been made by Donker[1] in 1926, by Stahly and Werkman[2] in 1942, and by Adams and Stanier[3] in 1945.

Stahly and Werkman[2] grew *B. polymyxa* in a medium containing 2 per cent glucose, 0.5 per cent peptone, 0.2 per cent dipotassium hydrogen phosphate, and 1 per cent calcium carbonate at a temperature of 30°C. For the fixation of aldehyde, 1 per cent calcium sulphite ($CaSO_3$) was used. Stahly and Werkman observed that the addition of acetaldehyde to the glucose medium resulted in larger yields of ethanol, acetylmethyl-carbinol (acetoin), and 2,3-butanediol, and that the addition of acetic acid resulted in larger yields of acetoin and 2,3-butanediol. They were therefore of the opinion that acetaldehyde was probably an intermediate in the fermentation. They also found that a low redox potential favored the production of 2,3-butanediol, whereas a high redox potential favored the production of acetoin.

Adams and Stanier[3] have prepared carbon balances for the anaerobic fermentations of glucose, xylose, pyruvic acid, and mannitol, using *B. polymyxa*, strain NRC 25. The following table summarizes some of the information obtained by them concerning these fermentations:

TABLE 110.—FERMENTATION OF GLUCOSE, XYLOSE, PYRUVIC ACID, AND MANNITOL BY *B. polymyxa*, STRAIN NRC 25[1]

Raw material	Weights, grams			
	Glucose	Xylose	Pyruvic acid	Mannitol
Amount raw material fermented	5 210	4 202	1 278	2.037
Amounts of end products formed				
2,3-butanediol	1 700	0 956	None	0 138
Acetoin	0 072	0 062	0 255	0 008
Ethanol	0 881	0 811	Trace	0.518
Acetic acid	0 050	0 129	0 455	0 132
Lactic acid			Trace	0 576
Succinic acid				0 075
Carbon dioxide	2 542	1 987	0 605	0 727
Hydrogen	0 041	0 046	0 016	0 038
Carbon recovery, per cent	101 6	92 9	93 0	106 8
CO_2 calculated/CO_2 observed	1 026	0 938	0 974	0.973
H_2 calculated/H_2 observed	1 079	0 712	0 950	0 930
O/R index	0 989	0 960	0 971	0 969

[1] ADAMS, G. A., and R. Y. STANIER, *Can. Jour. Research*, B, 23·1 (1945).

[1] DONKER, *op. cit.*

[2] STAHLY, G. L., and C. H. WERKMAN, *Biochem. Jour.*, 36: 575 (1942).

[3] ADAMS, and STANIER, *loc. cit.*

Study of the foregoing table will disclose interesting facts regarding the relative weights and ratios of the products formed by the fermentations of the respective raw materials—glucose, xylose, pyruvic acid, and mannitol. The 2,3-butanediol/ethanol ratio may be computed by comparing the moles of butanediol produced with those of ethanol. Since the molecular weight of 2,3-butanediol is 90 and that of ethanol is 46, there would be 90 g. of the former and 46 g of the latter produced when the ratio is 1/1.

The authors suggested that pyruvic acid was dissimilated anaerobically by *B. polymyxa* according to the following scheme:

$$CH_3 CO \; COOH + H_2O \rightarrow CH_3 COOH + CO_2 + H_2$$
<div align="center">Pyruvic acid   Acetic acid</div>

$$2CH_3 CO \; COOH \rightarrow 2CH_3 CHO + 2CO_2$$
<div align="center">Pyruvic acid   Acetaldehyde</div>

$$2CH_3 CHO \rightarrow CH_3 CO \; CHOH \; CH_3$$
<div align="center">Acetaldehyde   Acetoin</div>
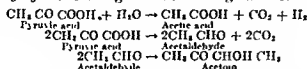
PRODUCTION OF ACETONE BY STRAINS OF *B. polymyxa*—Rose[1] confirmed the evidence presented by Donker[2] that acetone is produced from

TABLE III —FERMENTATION PRODUCTS OF TWO STRAINS OF *B polymyxa*[1,2]

Product	C2(3)	C25
Butanediol	45 5	40 6
Acetoin	2 8	0 2
Ethanol	76 7	83 2
Formic acid	1 2	2 4
Acetic acid	14 0	6 3
Lactic acid	3 2	2 9
Succinic acid	1 8	1 6
Malic acid	0 5	0 2
Carbon dioxide	188 3	200 7
Hydrogen	90 1	97 9
Acetone	Tr (?)	9 5
Dextrose fermented	5077	5066
Carbon recovery, per cent	97 2	98 3
CO_2, calc /obs	1 013	1 00
H_2, calc /obs	0 908	1 001
O/R index	0 987	1 001
$\dfrac{Acetoin + diol}{Ethanol}$ (by weight)	1 23	0 96

[1] Rose D *Can Jour Research,* F, **24.** 320 (1946)
[2] Yields expressed as millimoles per 100 millimoles of dextrose fermented

dextrose in small amounts by certain strains of *B. polymyxa* under anaerobic conditions The preceding table from the report by Rose supplies data concerning the products and amounts of each formed by the fermentation of dextrose by two different strains of *B polymyxa*.

[1] Rose, D, *Can Jour Research,* F, **24**: 320 (1946)
[2] Donker, *op cit*

3. *The Aeromonas Hydrophila Fermentation.*—This fermentation is characterized by the production of the levorotatory and meso forms of 2,3-butanediol, ethanol and lactic acid from sugars. It was investigated by Stanier and Adams,[1] of the Canadian National Research Council.

THE ORGANISM.—The fermentation described herein is brought about by *Aeromonas hydrophila*, a Gram-negative, nonsporeforming, rod-shaped organism of the family *Pseudomonadaceae*.

BASAL MEDIUM.—The basal medium used by Stanier and Adams contained the following ingredients: 1.25 g. of yeast extract, 1.0 g. of calcium carbonate, 0.025 g. of magnesium sulphate, 0.65 ml. of 1.0 M phosphate buffer (pH 7.4), and 250 ml. of tap water. The carbohydrates investigated were sterilized separately in concentrated aqueous solutions and were added to flasks containing the above basal medium (after their sterilization and at the time of inoculation). The concentration of carbohydrates used was 1 to 2 per cent.

FERMENTATION CONDITIONS.—Nitrogen gas (free from oxygen) was passed through each flask at a slow rate. The media were inoculated at 30°C. for 3 to 4 days.

PRODUCTS OF FERMENTATION.—The types of products formed and the quantities of each may be ascertained by reference to Table 112 which summarizes some data obtained by Stanier and Adams[1] in determining the carbon balances for the fermentation of glucose, xylose, and pyruvic acid by *Aeromonas hydrophila*.

FERMENTATION OF PYRUVIC ACID.—Stanier and Adams suggested that pyruvic acid was fermented by *Aeromonas hydrophila* in accordance with the three following reactions:

$$3CH_3 \cdot CO \cdot COOH + 3H_2O \rightarrow 3CH_3 \cdot COOH + 3CO_2 + 6H$$

$$\underset{\text{Pyruvic acid}}{} \qquad \underset{\text{Acetic acid}}{}$$

$$CH_3 \cdot CO \cdot COOH + 2H \rightarrow CH_3 \cdot CHOH \cdot COOH$$

$$\underset{\text{Lactic acid}}{}$$

$$4H \rightarrow 2H_2$$
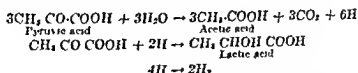
4. *The Bacillus Subtilis Fermentation.*—*Bacillus subtilis* (Ford's strain) produces 2,3-butanediol and glycerol as the principal end products when grown in a medium containing 3 per cent glucose, 1 per cent yeast extract, and 1 per cent calcium carbonate when the pH is maintained within the range of 6.0 to 6.8, when the temperature is 30°C. and when the conditions are anaerobic, according to Neish, Blackwood, and Ledingham.[2] The 2,3-butanediol appears to be a mixture of the levorotatory and meso forms in about equal quantities

The fermentation is quite complex in that the course of the fermenta-

[1] STANIER, R. Y., and G. A. ADAMS, *Biochem. Jour.*, 38: 168 (1944).

[2] NEISH, A. C., A. C. BLACKWOOD, and G. A. LEDINGHAM, *Science* 101: 245 (1945)

TABLE 112.—FERMENTATION OF GLUCOSE, XYLOSE, AND PYRUVIC ACID BY *Aeromonas hydrophila*[1]

	Glucose		Xylose		Pyruvic Acid	
	Weight, g	No. of moles/ 100 moles of glucose fermented	Weight, g	No. of moles/ 100 moles of xylose fermented	Weight, g	No. of moles/ 100 moles of pyruvic acid fermented
Amount of raw material fermented	5 490		4 010		3 530	
Amount of end product formed						
2,3-Butanediol	1 502	51 7	0 939	39 0	0 125	3 5
Acetoin	0 017	1 7	0 061	2 6	0 021	0 5
Ethanol	0 730	52 0	0 600	48 9	0 038	2 1
Acetic acid	0 085	4 6	0 150	9 3	1 800	74 8
Lactic acid	0 639	23 3	0 491	20 4	0 776	21 5
Succinic acid	0 130	3 6	0 036	1 1		
CO_2	2 230	166 2	1 584	131 7	1 470	83 8
H_2	0 035	57 5	0 029	53 9	0 035	43 6
Carbon recovery, per cent	98 2		96 6		106 3	
CO_2 calculated/CO_2 observed	0 997		1 011		1 013	
H_2 calculated/H_2 observed	1 108		1 126		1 102	
O/R index	1 021		0 988		1 032	
2,3-Butanediol ethanol (approx)	1 1		0 8 ⁣		1 6 ⁣	

[1] STANIER R Y., and G A ADAMS *Biochem Jour* 38 168 (1944)

tion may be sharply altered by what appear to be rather small changes in the conditions of fermentation, as for example, changes in the thiamin content of the medium, the pH, oxygen relationships, or perhaps in the strains of the organism used.[1]

HISTORICAL.—Desmots in 1901 reported that *B subtilis* formed acetoin when cultured on media rich in carbohydrates Lemoigne[2] in 1912 showed that *B. subtilis* produced 2,3-butanediol in the fermentation of

[1] NEISH, A. C., A C BLACKWOOD, and G A LEDINGHAM, *Can. Jour Research*, B, 23 : 290 (1945)
[2] LEMOIGNE, M , *Compt rendu* , 155 : 792 (1912)

carbohydrate-rich media and that acetoin was produced from the former by bacterial oxidation. Friedemann found that lactic acid was the chief product resulting from the fermentation of a medium composed of glucose, meat extract, and peptone, having an initial pH of 7.6 (buffered with phosphate). Acetic acid, formic acid, and ethanol were also formed. Gunsalus[1] has shown that thiamin exerts a very strong influence on the course of the fermentation. For example, he found that 70 to 90 per cent of the glucose of a medium was converted to lactic acid when the thiamin content was very small or absent. When thiamin was added to the nutrient glucose medium, 2,3-butanediol was formed in significant quantities (26 moles per 100 moles of glucose) and the yield of lactic acid dropped to 50 per cent based on the glucose. The foregoing facts will give some indication of the complexity of the fermentation. Neish, Blackwood, and Ledingbam studied extensively[2,3] the dissimilation of glucose by *B. subtilis* (Ford's strain).

THE ORGANISM.—Neish, Blackwood, and Ledingham[3] used in their studies the Ford strain of *B. subtilis* (N.C.T.C. 2586), which is stocked in the American Type Culture Collection as No. 9789.

THE INOCULUM.—*B. subtilis* was grown in nutrient broth at 30°C. for 24 hr. prior to use. Fermentation media were inoculated with 3 per cent of inoculum.

EFFECTS OF CONDITIONS OF FERMENTATION.—Fermentations were carried out by Neish, Blackwood, and Ledingham under a variety of conditions and with different results.

A medium containing 3 per cent glucose, 1 per cent yeast extract, and 1 per cent calcium carbonate at a temperature of 30°C., and at a pH of 6 8 to 6.2, appeared to be especially favorable for the production of 2,3-butanediol and glycerol when 300-ml. amounts in 1,000-ml. Erlenmeyer flasks were aerated with nitrogen gas at the rate of 100 ml per min. Under these conditions, 56 36 millimoles of 2,3-butanediol, 39 91 millimoles of glycerol, 19.06 millimoles of lactic acid, 12.88 millimoles of ethanol, and 5.56 millimoles of formic acid were produced per 100 millimoles of glucose dissimilated. Under the same conditions (except that the medium was aerated with bubbles of oxygen gas instead of nitrogen gas) 33.35 millimoles of 2,3-butanediol, 33.65 millimoles of acetoin, 7.38 millimoles of ethanol, 4.99 millimoles of acetic acid, 3.66 millimoles of glycerol, and small quantities of lactic, formic, and n-butyric acid were produced per 100 millimoles of glucose dissimilated. Carbon dioxide, but no molecular hydrogen, was produced from the glucose in each case.

[1] Gunsalus, I. C. *Jour. Bact.*, **48**: 261 (1944).
[2] . . . . 1: 290 (1945).
[3] . . .

The effect of pH on the dissimilation of glucose is shown in the following table. It will be noticed that at the higher pH range (7.6 to 6.8) there were decreases in the quantities produced of 2,3-butanediol and glycerol, and increases in the quantities of ethanol, lactic acid, succinic acid, formic acid, acetic acid, and n-butyric acid in comparison with the amounts produced of these same items at the lower pH range (6.2 to 5.8)

TABLE 113—EFFECT OF pH ON THE DISSIMILATION OF GLUCOSE[1,2,3]

Product	Millimoles per 100 millimoles of glucose fermented	
	Grown at pH 6.2 to 5.8	Grown at pH 7.6 to 6.8
2,3-Butanediol	56.15	36.16
Acetoin	Trace	Trace
Glycerol	26.28	16.39
Ethanol	18.21	28.70
Lactic acid	39.13	53.08
Succinic acid	Trace	5.05
Formic acid	9.97	30.14
Acetic acid	Doubtful	3.98
n-Butyric acid	Doubtful	2.76
Carbon dioxide (calc)	130.56	101.01
Carbon accounted for	100.0 per cent	97.0 per cent
Glucose dissimilated (4 days)	73.7 per cent	68.2 per cent

[1] NEISH A C, A C BLACKWOOD and G A LEDINGHAM, *Can Jour Research* B, 23:1290 (1945)
[2] *Medium* Glucose 3 per cent potassium dihydrogen phosphate 0.5 per cent dipotassium hydrogen phosphate 0.6 per cent magnesium sulfate heptahydrate 0.02 per cent casein hydrolysate 0.1 per cent.
[3] *Conditions* Grown at 30°C without aeration, the pH was measured with a glass electrode every 8 to 10 hr, and adjusted with N sodium hydroxide

In general, a high pH favored the production of acids and ethanol at the expense of the 2,3-butanediol and glycerol, while a low pH favored the production of 2,3-butanediol and glycerol.

A medium containing 1.0 per cent glucose, 1.1 per cent peptone, 0.3 per cent meat extract, and 1.5 per cent disodium hydrogen phosphate (Fredemann's medium), was fermented, without aeration at a temperature of 30°C and with an initial pH of 7.6 and a final pH of 6.0, resulted in the production of 98.50 millimoles of lactic acid and 20.43 millimoles of 2,3-butanediol per 100 millimoles of glucose dissimilated, according to Neish, Blackwood, and Ledingham.

5. *The Serratia marcescens Fermentation*   This fermentation was first studied by Pederson and Breed.[4] They used a medium that con-

[4] PEDERSON, C S and R S BREED, *Jour Bact* 16. 163 (1928)

carbohydrate-rich media and that acetoin was produced from the former by bacterial oxidation. Friedemann found that lactic acid was the chief product resulting from the fermentation of a medium composed of glucose, meat extract, and peptone, having an initial pH of 7.6 (buffered with phosphate). Acetic acid, formic acid, and ethanol were also formed. Gunsalis[1] has shown that thiamin exerts a very strong influence on the course of the fermentation. For example, he found that 70 to 90 per cent of the glucose of a medium was converted to lactic acid when the thiamin content was very small or absent. When thiamin was added to the nutrient glucose medium, 2,3-butanediol was formed in significant quantities (26 moles per 100 moles of glucose) and the yield of lactic acid dropped to 50 per cent based on the glucose. The foregoing facts will give some indication of the complexity of the fermentation. Neish, Blackwood, and Ledingham studied extensively[2,3] the dissimilation of glucose by *B. subtilis* (Ford's strain).

THE ORGANISM.—Neish, Blackwood, and Ledingham[3] used in their studies the Ford strain of *B. subtilis* (N.C.T.C. 2586), which is stocked in the American Type Culture Collection as No. 9789.

THE INOCULUM.—*B. subtilis* was grown in nutrient broth at 30°C. for 24 hr. prior to use. Fermentation media were inoculated with 3 per cent of inoculum.

EFFECTS OF CONDITIONS OF FERMENTATION.—Fermentations were carried out by Neish, Blackwood, and Ledingham under a variety of conditions and with different results.

A . . . . meat extract and
1 per

to 6 2, appeared to be especially favorable for the production of butanediol and glycerol when 300-ml. amounts in 1,000-ml. Erlenmeyer flasks were aerated with nitrogen gas at the rate of 100 ml. per min. Under these conditions, 56.36 millimoles of 2,3-butanediol, 39.91 millimoles of glycerol, 19.96 millimoles of lactic acid, 12.88 millimoles of ethanol, and 5.56 millimoles of formic acid were produced per 100 millimoles of glucose dissimilated. Under the same conditions (except that the medium was aerated with bubbles of oxygen gas instead of nitrogen gas) 33.35 millimoles of 2,3-butanediol, 33 65 millimoles of acetoin, 7.38 millimoles of ethanol, 4.99 millimoles of acetic acid, 3.66 millimoles of glycerol, and small quantities of lactic, formic, and n-butyric acid were produced per 100 millimoles of glucose dissimilated. Carbon dioxide, but no molecular hydrogen, was produced from the glucose in each case.

[1] GUNSALIS, I. C., *Jour. Bact.*, 48: 261 (1944).

[2] NEISH, BLACKWOOD, and LEDINGHAM, *Science*, 101: 245 (1945).

[3] NEISH, BLACKWOOD, and LEDINGHAM, *Can. Jour. Research*, B, 23: 290 (1945).

The effect of pH on the dissimilation of glucose is shown in the following table. It will be noticed that at the higher pH range (7.6 to 6.8) there were decreases in the quantities produced of 2,3-butanediol and glycerol, and increases in the quantities of ethanol, lactic acid, succinic acid, formic acid, acetic acid, and n-butyric acid in comparison with the amounts produced of these same items at the lower pH range (6 2 to 5.8).

TABLE 113 —EFFECT OF pH ON THE DISSIMILATION OF GLUCOSE[1,2,3]

	Millimoles per 100 millimoles of glucose fermented	
Product	Grown at pH 6 2 to 5 8	Grown at pH 7 6 to 6 8
2,3-Butanediol	56 15	36 16
Acetoin	Trace	Trace
Glycerol	26 28	16 39
Ethanol	18 21	28 70
Lactic acid	39 13	53 08
Succinic acid	Trace	5 05
Formic acid	9 97	30 14
Acetic acid	Doubtful	3 98
n-Butyric acid	Doubtful	2 76
Carbon dioxide (calc)	130 56	101 01
Carbon accounted for	100 0 per cent	97 0 per cent
Glucose dissimilated (4 days)	73 7 per cent	68 2 per cent

[1] NEISH A C A C BLACKWOOD and G A LEDINGHAM *Can Jour Research* B, **23:** 290 (1945)
[2] Medium Glucose 3 per cent potassium dihydrogen phosphate 0 5 per cent dipotassium hydrogen phosphate 0 6 per cent magnesium sulfate heptahydrate 0 02 per cent casein hydrolysate 0 1 per cent.
[3] Conditions Grown at 30°C without aeration, the pH was measured with a glass electrode every 8 to 10 hr and adjusted with N sodium hydroxide

In general, a high pH favored the production of acids and ethanol at the expense of the 2,3-butanediol and glycerol, while a low pH favored the production of 2,3-butanediol and glycerol.

A medium containing 1.0 per cent glucose, 1 1 per cent peptone, 0 3 per cent meat extract, and 1 5 per cent disodium hydrogen phosphate (Friedemann's medium), was fermented, without aeration at a temperature of 30°C and with an initial pH of 7 6 and a final pH of 6 0, resulted in the production of 98 50 millimoles of lactic acid and 20 13 millimoles of 2,3-butanediol per 100 millimoles of glucose dissimilated, according to Neish, Blackwood, and Ledingham

5. *The Serratia marcescens Fermentation* — This fermentation was first studied by Pederson and Breed.[1] They used a medium that con-

[1] PEDERSON, C. S., and R S BREED, *Jour Bact*, **16:** 163 (1928)

tained 1 per cent peptone broth and 3 per cent glucose, and which was buffered with an excess of sterile calcium carbonate. Using culture no. 1377 of *S. marcescens*, they obtained the following percentages of end products, based on the sugar fermented: 39.8 per cent lactic acid, 28 per cent carbon dioxide, 17.7 per cent acetoin and 2,3-butanediol, 5.5 per cent acetic acid, 4.7 per cent ethanol, 1.3 per cent succinic acid, 0.2 per cent

TABLE 114.—ANAEROBIC DISSIMILATION OF GLUCOSE BY *Serratia marcescens*[1]

Products	Millimoles of products per 100 millimoles of glucose dissimilated			
	Strain S1	Strain S2	Strain S3	Strain S9
2,3-Butanediol	57 90	55 20	51.45	42.45
Acetoin	0 25	0 50	0 81	1 14
Glycerol	6 14	4 18	4 54	5 63
Ethanol	40 85	41 30	42 24	25.90
Lactic acid	15 70	26.50	33 00	54.15
Formic acid	48 50	44 00	39 80	27.60
Acetic acid	Nil	Nil	Nil	Nil
Succinic acid	2 98	3 34	3 41	18 80
Carbon dioxide	103 8	102 5	106 1	78 2
Hydrogen	Nil	Nil	0 52	0 27
Fermentation time, days	17	12	9	7
Glucose dissimilated, per cent	93 2	88 5	99 5	99 9
Carbon accounted for, per cent	91 4	93 0	94 6	97 5
O/R index	0 99	1 03	1 04	1 01

[1] NEISH, A C., A C BLACKWOOD, F M ROBERTSON, and G. A LEDINGHAM, *Can Jour Research* B, 25: 65 (1947).

formic acid, and a small amount of hydrogen gas Similar end products were produced by other strains of *S. marcescens* and *S. indica*.

Neish and his associates[1] investigated fermentations carried out under anaerobic conditions, using four strains of *S. marcescens* that produced little or no hydrogen. They used a yeast extract medium (150 ml. in a 1-liter Erlenmeyer flask) that contained 5 per cent glucose, 0.5 per cent yeast extract, 0.05 per cent potassium dihydrogen phosphate, 0 05 per cent potassium monohydrogen phosphate, and 0.02 per cent magnesium sulphate. This medium was buffered with 2 per cent calcium carbonate separately sterilized. Anaerobic conditions were produced by continuously passing oxygen-free nitrogen through the medium. The

[1] NEISH, A C., A. C. BLACKWOOD, F M ROBERTSON, and G. A. LEDINGHAM, *Can Jour Research.* B, 25: 65 (1947)

fermentations were carried out at 35°C. Results obtained are shown in Table 114

Clarification of Mashes.—Strohmaier and Lovell[1] investigated the clarification of acid-hydrolyzed corn mashes and the fermented mashes of the same type (glycol beer) by filtration and centrifugation methods They found that pH and temperature were very important factors in connection with clarification The optimum pH depended upon the nature of the mash and was 5.0 and 6 2 with cooked (continuous process), unfermented mash and 4 8 for glycol beer produced on a pilot-plant scale The rates of filtration were highest, at a constant pH, at the highest temperature (210°F)

The clarification of corn mashes by both filtration and centrifugation could be facilitated by adding small amounts of a swelling type of bentonite to the mashes at a pH of 2 0 to 3 0 and at high temperatures, according to the above investigators

Recovery of 2,3-Butanediol.—It is not a simple matter to recover 2,3-butanediol from the fermentation liquor (beer), on account of its high boiling point (about 180°C), its suspended and soluble solids, and the presence of nonvolatile substances

Blom, Reed, Efron, and Mustakas[2] described a process for recovering 2,3-butanediol from fermentation liquors In their process, the beer is concentrated to a sirup in evaporators and then steam-stripped at an elevated pressure in a special column packed with ceramic balls

The recovery of 2,3-butanediol from glycol beer as formal has been described by Senkus.[3] A flow sheet for glycol recovery by his method is shown in Fig 70.

In the laboratory, the fermented medium may be saturated with potassium carbonate and then extracted with *n*-butanol, or continuously extracted with diethyl ether by the method of Kolfenbach, Kooi, Fulmer, and Underkofler[4]

Other methods for recovering 2,3-butanediol have been reported by Liebmann,[5] Othmer, Bergen, Shlechter, and Bruins,[6] Rose and King;[7] and others

[1] STROHMAIER, A J , and C L LOVELL, *Ind Eng Chem* , **38** : 721 (1946)

[2] BLOM, R H , D L REED, A EFRON, and G C MUSTAKAS, *Ind Eng Chem* , **37** : 865 (1945)

[3] SENKUS, M , *Ind Eng Chem* , **38** : 913 (1946)

[4] KOLFENBACH, J J , E R KOOI, E I FULMER, and L A UNDERKOFLER, *Ind Eng Chem* , *Anal Ed* , **16** : 473 (1944)

[5] LIEBMANN, A J , *Oil & Soap*, **22** : 349 (1945)

[6] OTHMER, D F , W S BERGEN, N SHLECHTER, and P F BRUINS, *Ind Eng Chem* , **37** : 890 (1945)

[7] ROSE and KING, *loc cit*

RAW AND PRODUCTIVE MATERIALS

2,3-BUTANEDIOL RECOVERY FROM FERMENTATION LIQUOR
BASIS: 3,000 LB. BEER CONTAINING 3.5% GLYCOL

PRODUCTS AND BY-PRODUCTS

25,000 GALLONS COOLING WATER 20°C.

3,000 LB. BEER 3.5 % 2,3-BUTANEDIOL 0.2 % ETHANOL, ALSO ACETONE AND DIACETYL

40 GALLONS TAILS CUT FROM PREVIOUS RUN (8-10 % FORMAL)

101 LB. SULFURIC ACID

101 LB. FORMALDEHYDE

140 GALLONS METHANOL

14,000 LB. STEAM

28 LB. CONDENSATE WATER (TO GLYCOL RECOVERY)

211 LB. CAUSTIC (TO GLYCOL RECOVERY)

REACTION STILL
1,200-GALLON
30-PLATE STEEL COLUMN
ETHYL ALCOHOL RECOVERY

PREPARATION AND DISTILLATION OF GLYCOL FORMAL

CONDENSER

GLYCOL REGENERATION

CONDENSER

METHYLAL HYDROLYSIS

FORMALDEHYDE RECOVERY

ETHYL ALCOHOL 120 LB. 20% ALCOHOL SOLUTION

2,3-BUTANEDIOL 105 LB.

27 LB. (8-10 % FORMAL) 40 GALLONS TAILS CUT (RECYCLE)

RECOVERED METHANOL 333 GALLONS

RECOVERED FORMALDEHYDE 90 LB.

2.7 LB. SODIUM SULFATE

3,000 LB. STILL RESIDUE 10% SULFURIC ACID

14,000 LB. STEAM CONDENSATE

25,000 GALLONS EXIT COOLING WATER 40°C.

Fig. 70.—Flow sheet for glycol recovery. [Courtesy of M. Senkus, Ind. Eng. Chem., 38: 913 (1946).]

Determination of 2,3-Butanediol.—The quantitative determination of the amount of 2,3-butanediol in a sample may be accomplished by the periodate oxidation method, which was developed by Johnson, at the Department of Biochemistry at the University of Wisconsin.[1] The 2,3-butanediol is oxidized by periodic acid to form 2 molecules of acetaldehyde. The aldehyde is fixed or absorbed by bisulphite. The resultant aldehyde-bisulphite addition product is treated with alkali and the bisulphite determined by titration with a dilute iodine solution.

Uses.—The uses of various forms of 2,3-butanediol have been discussed by Liebmann[2] and reviewed generally by Underkofler and Fulmer.[3] Reference has already been made to its potential use as a source of synthetic rubber. In this process, the 2,3-butanediol may be converted to 1,3-butadiene by a suitable method, such as by the pyrolysis of the diacetate. The levorotatory form [d-(−)-2,3-butanediol] is a satisfactory antifreeze agent, according to Clendenning.[4] He and Wright[5] reported that a mixture of 20 per cent of methanol, 40 per cent of butanediol, and 40 per cent of water was suitable for an antifreeze at temperatures as low as minus 50°C. Neish and MacDonald[6] described the preparation and physical properties of cyclic acetals and ketals that were readily derived from levorotatory 2,3-butanediol. The 2,3-butanediols (and their derivatives) have potential uses as solvents, moisteners, and softeners, as has been mentioned previously.

References on the Production and Properties of 2,3-Butanediol

ADAMS, G. A. Production and Properties of 2,3-Butanediol VII. Fermentation of Wheat by *Aerobacillus polymyxa* under Aerobic and Anaerobic Conditions, *Can Jour. Research*, F, 24: 1–11 (1946).

—— and J. D. LESLIE. Production and Properties of 2,3-Butanediol VIII. pH Control in *Aerobacillus polymyxa* Fermentation and Its Effects on Products and Their Recovery, *Can Jour Research*, F, 24: 12–28 (1946).

—— and ——. Production and Properties of 2,3-Butanediol X. The Effects of Surface-Volume Ratio and Reduced Pressure on the Fermentation of Carbohydrates by *Aerobacillus polymyxa* and *Aerobacter aerogenes*, *Can Jour Research*, F, 24: 107–118 (1946).

—— and R. Y. STANIER. Production and Properties of 2,3-Butanediol. III Studies on the Biochemistry of Carbohydrate Fermentation by *Aerobacillus polymyxa*, *Can. Jour. Research*, F, 23: 1–9 (1945).

[1] JOHNSON, M. J., *Ind. Eng. Chem.*, *Anal. Ed.*, 16: 626 (1944).

[2] LIEBMANN, A. J., *Oil & Soap*, 22: 31 (1915).

[3] UNDERKOFLER, L. A., and E. I. FULMER, *Wallerstein Labs Communs*, 11 (No. 32): 41 (1918).

[4] CLENDENNING, K. A., *Can Jour Research*, F, 24: 249 (1946).

[5] CLENDENNING, K. A., and D. E. WRIGHT, *Can. Jour. Research*, F, 24: 287 (1946).

[6] NEISH, A. C., and F. J. MACDONALD, *Can. Jour. Research*, B, 25: 70 (1947).

BLACKWOOD, A. C., and G. A. LEDINGHAM: Production and Properties of 2,3-Butane-diol. XX. Influence of the Inoculum on the *Aerobacillus polymyxa* Fermentation, *Can. Jour. Research*, F, **25**: 180–191 (1947).

BLOM, R H , D L. REED, A. EFRON, and G. C. MUSTAKAS: Recovery of 2,3-Butylene Glycol from Fermentation Liquors, *Ind. Eng. Chem.*, **37**: 865–870 (1945).

BÖESEKEN, J , and R. COHEN: La Configuration des Butanediols 2.3, *Rec. trav. chim ,* **47**: 839–848 (1928).

BREDEN, C R : The Chemical Action of *Aerobacter faeni* on Xylose and on Sucrose, Doctoral Thesis, Iowa State College, 1930.

CHRISTENSEN, L M , Increased Yield of 2,3-Butylene Glycol, U.S Patent 2,314,025, Mar 14, 1911

CLENDENNING, K A : Production and Properties of 2,3-Butanediol. XI. Evaluation of *Levo*- 2,3-Butanediol as a Non-Volatile Antifreeze Compound, *Can Jour. Research*, F, **24**: 249–271 (1946).

———: Production and Properties of 2,3-Butanediol. XVI. Density, Optical Rotatory Power, and Refraction of Aqueous 2,3-Butanediol Solutions, *Can Jour. Research*, B, **24**: 269–279 (1946)

——— and D E WRIGHT: Production and Properties of 2,3-Butanediol XII Antifreeze Properties of Ternary Aqueous Solutions Containing *Levo*-2,3-Butane-diol as a Major Component, *Can. Jour. Research*, F, **24**: 287–299 (1946)

DONKER, H. J. L.: "Bijdrage tot de kennis der boterzuurbutylalcohol en acetongist-ingen," Thesis, Delft, 1926.

ELDER, A L.: Progress of Butadiene Production, *Ind. Eng. Chem.*, **34**: 1200–1206 (1942).

FRATKIN, S. B., and G A. ADAMS: Production and Properties of 2,3-Butanediol IX The Effect of Various Nutrient Materials on the Fermentation of Starch by *Aerobacillus polymyxa*, *Can. Jour. Research*, **24**: 29–38 (1946).

FULMER, E. I.: The Physico-Chemical Approach to Problems of Fermentation, *Brewers Digest*, **18** (No. 8): 29–32; (No. 9): 37–39, 44 (1943)

——— A C. BANTZ, and L. A. UNDERKOFLER: The Use of Alfalfa Extract to Supply Nutrients for the Growth and Chemical Activities of *Aerobacter suboxydans*, *Iowa State Coll , Jour. Sci.*, **18**: 369–376 (1944)

FULMER, E I , L. M. CHRISTENSEN, and A R. KENDALL: Production of 2,3-Butylene Glycol by Fermentation. Effect of Sucrose Concentration, *Ind Eng. Chem ,* **25**: 798–800 (1933)

——— L. A UNDERKOFLER, and A. C. BANTZ: The Production of Acetylmethyl-carbinol by the Action of *Acetobacter suboxydans* upon 2,3-Butylene Glycol, *Jour Am. Chem. Soc ,* **65**: 1425–1427 (1943).

. . . . Strain of *Bacillus*
. . . . *genes* (Esche-
rich) on Glucose and Mannitol: Production of 2,3-butylene . . l and Acetyl-methylcarbinol, *Proc. Roy Soc. (London)*, Series B, **77**: 399–405 (1906).

HILL, R., and E. ISAACS: British Patent 483,989, Apr. 28, 1938

JOHNSON, M J.: Determination of 2,3-Butylene Glycol in Fermentations, *Ind Eng Chem , Anal. Ed ,* **16**: 626–627 (1944)

KATZNELSON, H : Studies with *Bacillus polymyxa*. I Some Factors Affecting the Fermentation of Wheat by *Bacillus polymyxa*, *Can. Jour. Research*, C, **22**: 235–240 (1944).

———: Studies with *Bacillus polymyxa*. II. Bacteriophage of *Bacillus polymyxa* in Relation to the 2,3-Butanediol Fermentation, *Can Jour. Research*, C, 22: 241–250 (1944)

———: Studies with *Bacillus polymyxa*. IV Nitrogen Requirements in Relation to 2,3-Butanediol Production from Starch, *Can Jour Research*, C, 24: 99–108 (1946).

——— and A. G LOCHHEAD: Studies with *Bacillus polymyxa* III Nutritional Requirements, *Can Jour. Research*, C, 22: 273–279 (1944)

KENDALL, A R · "The Biochemistry of the Production of 2,3-Butylene Glycol by Fermentation," Doctor's Thesis, Iowa State College, 1934

KOLFENBACH, J J, E R KOOI, E I FULMER, and L A UNDERKOFLEN Laboratory Continuous Countercurrent Liquid—Liquid Extractor, *Ind Eng Chem*, *Anal.* *ed*, 16: 473–474 (1944).

KOOI, E R Production of the 2,3-Butanediol by the Fermentation of Starch, *Iowa State Coll Jour Sci*, 21: (No 1)· 36–37 (1946)

———, E I FULMER, and L A UNDERKOFLER. Production of 2,3-Butanediol by Fermentation of Cornstarch, *Ind Eng Chem*, 40 (No 8)· 1440–1445 (1948).

KLUYVER, A J, and M A SCHEFFER: Process for the Preparation of 2,3-Butylene-Glycol from Carbohydrates, U S Patent 1,899,156, Feb 28, 1933, also Canadian Patent 296,070, Dec 24, 1929

LEDINGHAM, G A, and G A ADAMS Canadian Patent 435,716, July 9, 1946

———, ——— and R Y STANIER: Production and Properties of 2,3-Butanediol, I Fermentation of Wheat Mashes by *Aerobacillus polymyxa*, *Can Jour. Research*, F, 23: 48–71 (1945).

LEES, T M, E I FULMER, and L A UNDERKOFLER· The Fermentation of the Stereoisomeric 2,3-Butanediols by *Acetobacter suboxydans*, *Iowa State Coll. Jour Sci*, 18: 359–367 (1944)

LESLIE, J D, and A CASTAGNE Production and Properties of 2,3-Butanediol XIV Butanediol Analysis for Process Control, *Can Jour Research*, F, 24: 311–319 (1946)

MORELL, S A, and A H AUERNHEIMER· Configuration of the 2,3-Butylene Glycols, *Jour Am Chem Soc* 66: 792–796 (1944)

——— H H GELLER, and E C LATHROP. Conversion of 2,3-Butylene Glycol to 1,3-Butadiene by Pyrolysis of Diacetate, *Ind Eng Chem*, 37: 877–884 (1945)

NEISH, A C Kinetics of the Catalytic Dehydrogenation of Alcohols and Glycols in the Gaseous State, *Can Jour Res*, B, 23: 49–69 (1945)

——— Production and Properties of 2,3-Butanediol IV Purity of the Levorotatory 2,3-Butanediol Produced by *Aerobacillus polymyxa Can Jour. Res*, B, 23: 10–16 (1945)

——— Production and Properties of 2,3-Butanediol XXIII Condensation of the Isomeric 2,3-Butanediols with Ethyl Acetoacetate, *Can Jour Res*, B, 25: 423–429 (1947)

———, A C BLACKWOOD, and G A LEDINGHAM A 2,3-Butanediol-Glycerol Fermentation, *Science*, 101: 215 (1945)

———, ——— and ——— Dissimilation of Glucose by *Bacillus subtilis* (Ford's Strain), *Can Jour Research*, B, 23: 290–296 (1945)

———, F M ROBERTSON, and G A LEDINGHAM, Production and Properties of 2,3-Butanediol XVIII Dissimilation of Glucose by *Serratia marcescens*, *Can Jour Research*, B, 25: 65–69 (1947)

Neish, A. C., V. C. Haskell, and F. J. Macdonald: Production and Properties of 2,3-Butanediol. VI. Dehydration by Sulphuric Acid, Can. Jour. Res., B, 23: 281–289 (1945).

———, ———, and ———: Production and Properties of 2,3-Butanediol. XXI. Catalytic Cracking of Cyclic Acetals and Ketals Derived from 2,3-Butanediol. Can. Jour. Res., B, 25: 266–271 (1947).

——— and F. J. Macdonald: Production and Properties of 2,3-Butanediol. XIX. Cyclic Acetals and Ketals Derived from Levo-2,3-Butanediol, Can. Jour. Res., B 25: 70–79 (1947).

N V. Nederlandsche Gist en Spiritusfabriek: Holland Patent 33,878, Nov. 10, 1934.

Olson, B. H: "Production of 2,3-Butylene Glycol by Aerobacter aerogenes," Thesis, University of Wisconsin, 1945.

——— and M. J. Johnson: The Production of 2,3-Butylene Glycol by Aerobacter aerogenes 199, Jour. Bact, 55 (No. 2): 209–222 (1948).

Othmer, D. F., W. S. Bergen, N. Shlechter, and P. F. Bruins: Liquid-Liquid Extraction Data, Systems Used in Butadiene Manufacture from Butylene Glycol, Ind. Eng. Chem., 37: 890–894 (1945).

———, N. Shlechter, and W. A. Koszalka: Composition of Vapors from Boiling Binary Solutions, Systems Used in Butadiene Manufacture from Butylene Glycol, Ind. Eng. Chem., 37 (No. 9): 895–900 (1945).

Paretsky, D., H. G. Wood, and C. H. Werkman: Bacterial Oxidation of 2,3-Butylene Glycol, Jour. Bact., 44 (No. 2): 257 (1942)

Pederson, C. S., and R. S. Breed: The Fermentation of Glucose by Organisms of the Genus Serratia, Jour. Bact., 16: 163–185 (1928)

Perlman, D.: Production of 2,3-Butylene Glycol from Wood Hydrolyzates, Ind. Eng. Chem., 36: 803–804 (1944).

Porter, R., C. S McCleskey, and M. Levine: The Facultative Sporulating Bacteria Producing Gas from Lactose, Jour. Bact., 33: 163–183 (1937)

Robertson, F M., and A. C. Neish: Production and Properties of 2,3-Butanediol. XXIV. The Cyclic Sulphates of meso- and levo-2,3-Butanediol, Can. Jour. Research, B, 25: 491–493 (1947).

Rose, D.: Production and Properties of 2,3-Butanediol XXII. The Effect of Aerobic Conditions on the Aerobacillus Fermentation, Can. Jour. Research, F, 25: 273–279 (1947).

——— and W S. King: Production and Properties of 2,3-Butanediol V. Small-Scale Production Unit, Can. Jour. Research, F, 23: 79–89 (1945).

Scheffer, M. A : "De suikervergisting door bacteriën der colgroep," Thesis, Delft, 1928.

———: Microbiological Preparation of 2,3-Butylene Glycol, U.S. Patent 2,064,339, Dec. 15, 1936.

Schnepf, L. E, J W Dunning, H. H Geller, S A. Morell, and E C Lathrop: Pilot-plant Conversion of 2,3-Butylene Glycol Diacetate to 1,3-Butadiene, Ind. Eng. Chem , 37: 884–889 (1945).

Senkus, M.: Recovery of 2,3-Butanediol Produced by Fermentation, Ind Eng Chem, 38: 913–916 (1946)

Silverman, M.: The Microbiological Formation of Acetylmethylcarbinol, Iowa State Coll. Jour Sci , 17: 120–122 (1942)

——— and C. H. Werkman: The Formation of Acetylmethylcarbinol from Pyruvic Acid by a Bacterial Enzyme Preparation, Jour. Biol. Chem., 138: 35–48 (1941)

SLADE, H D, and C H. WERKMAN: Assimilation of Acetic and Succinic Acids Containing Heavy Carbon by *Aerobacter Indologenes*, *Arch. Biochem*, 2: 97–111 (1943).

SLOTTER, R L, and C T LANGFORD: Wheat Starch Manufacture, *Ind. Eng Chem.*, 36: 404–408 (1944)

STAILY, G L, and C. H WERKMAN: Origin and Relationship of Acetylmethylcarbinol to 2,3-Butylene Glycol in Bacterial Fermentations, *Biochem. Jour.*, 36: 575–581 (1942)

STANIER, R. Y, and G A ADAMS The Nature of the *Aeromonas* Fermentation, *Biochem Jour*, 38: 168–171 (1944)

——, G A. ADAMS, and G A LEDINGHAM: Production and Properties of 2,3-Butanediol   II Strains of *Aerobacillus polymyxa* in Relation to Filterability and Butanediol Production, *Can Jour. Research*, F, 23: 72–78 (1945)

—— and S B FRATKIN: Studies on the Bacterial Oxidation of 2,3-Butanediol and Related Compounds, *Can Jour Research*, B, 22: 140–153 (1944)

STROHMAIER, A. J, and C L. LOVELL: Clarification of Acid-Hydrolyzed Mash and Beer for the Production of 2,3-Butanediol from Corn, *Ind Eng Chem.*, 38: 721–724 (1946).

TOLLEFSON, E L., J A WHEAT, and J D LESLIE: Production and Properties of 2,3-Butanediol.   XIII. Purification of By-Product Ethanol, *Can Jour Research*, F, 24: 300–310 (1946)

UNDERKOFLER, L A, and E I FULMER: The Production of 2,3-Butylene Glycol by Fermentation, *Wallerstein Labs Communs*, 11 (No. 32): 41–51 (1948)

VERHAVE, T. H . Brit Patent 315,263, July 10, 1928.

—— French Patent 676,876, June 19, 1929

——. Brit Patent 335,280, June 25, 1929.

—— German Patent 572694, Mar 25, 1933

WALPOLE, G S : The Action of *Bacillus lactis aerogenes* on Glucose and Mannitol Part II   The Investigation of the 2 3-Butanediol and the Acetylmethylcarbinol Formed, the Effect of Free Oxygen on Their Production, the Action of *B. lactis aerogenes* on Fructose, *Proc. Roy Soc. (London)*, Ser. B, 83: 272–286 (1911).

WARD, O. E, O. G. PETTIJOHN, and R D. COGHILL: Production of 2,3-Butanediol from Acid-Hydrolyzed Starch, *Ind Eng. Chem*, 37: 1189–1194 (1945)

——, ——, L B LOCKWOOD, and R D COGHILL: Optical Isomers of 2,3-Butanediol Produced by Fermentation, *Jour. Am Chem Soc*, 66: 541–542 (1944)

——, L J WICKERHAM, O G PETTIJOHN, and L B LOCKWOOD: Levo-2,3-Butylene Glycol and a Method for Producing the Same, U S. Pat 2,359,950, Oct 10, 1944.

WERKMAN, C H   An Improved Technic for the Voges-Proskauer Test, *Jour. Bact*, 20: 121–125 (1930)

SOME MINOR BACTERIAL ACTIVITIES WITH INDUSTRIAL IMPLICATIONS

Our consideration of the possibilities of utilizing bacteria industrially would be unnecessarily incomplete if no mention was made of a number of processes, somewhat less sharply defined than those which have preceded, but which have well-grounded industrial implications. Through further research some of these minor bacterial activities may assume considerably greater significance, although they may not supply a basic reaction for an industry and may not yield specific end products that can be merchandised in pure form or in large quantity. Some of the bacterial processes here may prove to be contributory and useful as adjuncts in other industries. Others may aid the student to a somewhat clearer conception of processes long used in industry but in which the part played by microbic agencies has not been well defined. In such cases it is still impossible to draw conclusions or make statements that can be regarded as fixed and final

ENZYME PRODUCTION BY BACTERIA

Active enzyme production is recognized as a qualification of many kinds of bacteria, but the field of industrial application has not been thoroughly investigated. As has been already pointed out in earlier chapters, bacteria produce several enzymes, including those which can hydrolyze carbohydrates, proteins, and fats and otherwise break down complex substances. The first step in the production of enzymes is to secure cultures of bacteria that will grow profusely and produce most efficiently the particular enzyme desired. Cultural conditions, such as the composition and pH of the medium, the use of sterile equipment and media, the temperature, the use of aeration, and other factors must be carefully worked out and controlled.

Amylases. *Production Studies.*—The production of amylase by bacteria has been studied by a number of scientists. Boidin and Effront[1] investigated amylase production by *Bacillus subtilis* and *B. mesentericus.*

[1] BOIDIN, A., and J. EFFRONT, U.S Patent 1,227,374, May 22, 1917, and U S Patent 1,227,525, May 22, 1917

Wallerstein,[1] and Schultz, Atkin, and Frey[2] have described methods for preparing media for use in producing bacterial amylase. Janke and Schaefer[3] have investigated amylase production by species of the genus *Bacillus* and two species of the genus *Sarcina*; Tilden and Hudson,[4] by *B. macerans* and *B. polymyxa*; and Hockenhull and Herbert,[5] by *Clostridium acetobutylicum*. Bois and Savary[6] described the properties of the amylase produced by organisms of the genera *Actinomyces* and *Phytomonas*. Beckord, Kneen, and Lewis[7] have described the production of bacterial amylases on wheat bran; and Beckord, Peltier, and Kneen,[8] on thin stillage. Peltier and Beckord,[9] Kneen and Sandstedt,[10] and Kneen and Beckord[11] have studied a large number of bacterial isolates to determine the quantity and quality of amylase produced.

Wallerstein Procedure.—Wallerstein[12] has described a procedure for producing these bacterial enzymes, in particular, amylases. The medium used may contain starch or the products of an enzymatically digested starch; nitrogen from casein or from soybean or peanut cakes or other sources, hydrolyzed by means of enzymes or acids; mineral salts, such as phosphates, and potassium, calcium, and magnesium salts plus traces of iron and manganese; and water. The medium, after filtration, sterilization, and cooling, is inoculated with a strain of *B. subtilis*. Best results are obtained when the inoculated medium is poured to form shallow layers in trays (contained in large culture vessels, each of which may have a capacity for as much as 1,000 gal.), when the incubation temperature is about 30°C, and when aeration is supplied. Temperature and aeration must be controlled carefully, the aeration being strongest at the beginning. Growth appears as bacterial films.

After the maximum enzyme content has been obtained, in perhaps a week, the culture medium containing the bacterial cells is centrifuged at approximately 14,000 r.p.m. to remove the bacteria. The resultant liquor is preserved by incubation at a low temperature or by the use of chemical antiseptics.

[1] WALLERSTEIN, L., *Ind. Eng. Chem.*, **31**: 1218 (1939).
[2] SCHULTZ, A., L. ATKIN, and C. N. FREY, U.S. Patent 2,159,678, May 23, 1939.
[3] JANKE, A., and R. SCHAEFER, *Zentr. Bakt. Parasitenk.*, **II**, 102: 211 (1940).
[4] TILDEN, E. B., and C. S. HUDSON, *Jour. Bact.*, **43**: 527 (1942).
[5] HOCKENHULL, D. J. D., and D. HERBERT, *Biochem. Jour.*, **39**: 102 (1945).
[6] BOIS, E., and J. SAVARY, *Can. Jour. Research*, **B,23**: 208 (1945).
[7] BECKORD, L. D., R. KNEEN, and L. H. LEWIS, *Ind. Eng. Chem.*, **37**: 692 (1945).
[8] BECKORD, L. D., G. L. PELTIER, and R. KNEEN, *Ind. Eng. Chem.*, **38**: 232 (1946).
[9] PELTIER, G. L., and L. D. BECKORD, *Jour. Bact.*, **50**: 711 (1945).
[10] KNEEN, E., and R. M. SANDSTEDT, *Arch. Biochem.*, **9**: 235 (1946).
[11] KNEEN, E., and L. D. BECKORD, *Arch. Biochem.*, **10** (No. 1): 41 (1946).
[12] WALLERSTEIN, *loc. cit.*

Waldmann Process.—Waldmann[1] has described a process for produc-ing amylolytic enzymes from mashes containing carbohydrate and nitro-gen. Bacteria of the *B. mesentericus* and *B. subtilis* groups are used to inoculate mashes that contain at least 40 parts of carbohydrate-con-taining substances, such as starch, soluble starch, or starch decomposition products, for 1 part of assimilable nitrogen, such as ammonium sulphate, ammonium lactate, or other ammonium salts. Buffers, as for example a mixture of KH_2PO_4 and K_2HPO_4 are added to the mash to maintain the pH level between 7 and 8 during the growth period. Air, in a fine state of subdivision, is passed through the entire mash for 2 days, after which the enzyme solution is clarified and filtered. Concentration is effected in a vacuum apparatus. It is stated that 1 kg. of the original enzyme solution is capable of converting 1,000 to 3,000 kg. of starch.

Waldmann[1] cited an example of the process in his patent. In a vat of 1,000-hectoliter capacity are sterilized with pressure 700 hectoliters of a 3 per cent solution of starch that contains 0.02 per cent of nitrogen as ammonium sulphate. Eight parts of a mixture of KH_2PO_4 and K_2HPO_4 of the correct proportions to produce and maintain a pH of 7.1 in the solution, are added (during the cooling process) to the vat for each 1,000 parts of the starch solution. After the mash has been cooled to 28°C., it is inoculated with amylase-forming bacteria of the mesentericus group and then aerated for 2 days, after which it is clarified and filtered.

The vat may be constructed of aluminum or another metal protected by a coating of rubber or otherwise treated so as to render it inactive.

The Production of Bacterial Amylases on Wheat Bran.—Beckord, Kneen, and Lewis[2] have described a laboratory procedure for producing bacterial amylases on wheat bran, which is as follows:

A mash may be prepared by adding 2.5 parts of dilute *phosphate buffer* (1.5 g. of KH_2PO_4 and 3.5 g. of $K_2HPO_4 \cdot 3H_2O$ per liter) to 1 part by weight of wheat bran (or other combination) in a suitable container and autoclaving the mixture for 1 hr. at about 121°C. The pH of the mash is initially approximately 6.0. The sterilized mash is inoculated with a suspension of isolate No. 23 of *B. subtilis*, incubated at 37°C. for 48 hr. and then extracted with the phosphate buffer described above for 1 hr. at 30°C. There is added 1 ml. of a 20 per cent $CaCl_2 \cdot 2H_2O$ solution for each 40 ml. of buffer. The precipitate and bran are separated by centrifuging, yielding a clear enzyme-containing extract.

The conditions of production were studied in further detail by Beck-ord and his associates.[2] They found that isolate No. 23 was the best of

[1] WALDMANN, R., U.S. Patent 2,302,079, Nov. 17, 1942.
[2] BECKORD, KNEEN, LEWIS, *loc. cit.*

16 aerobic bacilli known to hydrolyze starch; that neither the concentration of the inoculum nor its age had appreciable influence on amylase production; that subculturing more than once in a liquid mash did not stimulate production; and that amylase production was dependent chiefly upon the concentration of nutrient (wheat bran) present, the highest yield of amylase per unit weight of bran resulting when 1 part of bran to 1.75 parts of phosphate buffer was used.

The advantages of producing amylases on wheat bran are (1) the large surface area available for the growth of the organism, and (2) the fact that the material may be air-dried rapidly and rendered relatively stable.[1]

*Production from Thin Stillage —*Beckord, Peltier, and Kneen[2] have reported on the laboratory-scale production of amylases from thin stillage. The latter is obtained as a by-product of the ethyl alcohol fermentation of converted grains and contains water solubles derived from the malt-saccharified grains and the malt, grain fines, and non-viable yeast cells.

The organism employed for amylase production by Beckord and his associates[2] was a strain of *B subtilis* designated as Isolate 23, which has been described by Beckord, Kneen, and Lewis[3] This bacillus characteristically produced a pellicle on thin stillage. Amylases were produced by the bacteria making up the pellicle and secreted into the medium directly below. Most of the enzymes were to be found in a rather narrow zone immediately below the pellicle unless the medium was agitated.

Isolate 23 was cultured on peptone–beef-extract-agar slants. A loopful of the bacterial growth was used to inoculate a 50-ml. portion of sterile thin stillage (at a pH of 7 to 8) contained in a screw-capped, glass bottle (6 × 6 × 14 cm). After incubation at 37°C for 24 hr this culture was used to inoculate the medium used for amylase production or to inoculate a second subculture.

Beckord and his associates[2] produced amylases from thin stillage by several methods: a shallow pan method, an agitation method, and a drip method In each case, the stillage was neutralized with sodium carbonate in such manner that the pH after sterilization was 7 to 8.

In the shallow-pan method, the sterilized thin stillage in layers 2.5 to 3 8 cm. deep was inoculated and incubated in an undisturbed condition for 6 days. It was found that amylase activity (based on the dextrinization time in minutes at 30°C [4] began to decrease after 6 days.

[1] *Ibid.*
[2] Beckord, L. D , G L Peltier, and E. Kneen, *Ind Eng Chem* , 38 : 232 (1946).
[3] Beckord, Kneen, and Lewis, *loc cit*
[4] *Ibid*

In the agitation method, portions of thin stillage too deep for efficient amylase production by the surface culture (quiescent) method were inoculated with the thin stillage culture of *B. subtilis* (Isolate 23) and incubated at room temperature for 24 hr. Then a stirrer was put into operation which agitated the medium below the pellicle without disturbing the pellicle. This, of course, brought fresh medium into contact with the pellicle and removed metabolic products. A control, using the shallow-pan method, was carried out at the same time. This method was superior to the quiescent method.

In the drip method, devised by Beckord and his collaborators, sterile thin stillage was permitted to drip at a controlled rate down over a column of moist poplar chips in a glass tube 60 mm. in diameter and 1.5 meters long. The top of the tube contained a rubber stopper through which four inlet tubes conducted the stillage to the top of the column, and an air outlet. The bottom of the tube likewise contained a rubber stopper with an air inlet tube and a stillage outlet tube. The apparatus was sterilized with steam at atmospheric pressure for 3 hr. before use. After cooling, the chips were inoculated with 500 ml. of a thin stillage culture grown for 24 hr. at 30°C. The tube was incubated for 24 hr. at room temperature to encourage the development of the organisms on the chip surfaces Six liters of sterilized wheat-corn stillage at a pH of 7 were allowed to drip through the four inlet tubes and down over the chips at a controlled rate of 3 liters per day. Sterile air was passed up through the tube The amylase activity of the portion collected after the first day of flow was equal to that obtained in 5 days by the quiescent medium method. The activity of the portion collected on the second day was of the same value However, repassage of these portions through the tube resulted in a product of lower activity.

Attempts to produce amylase by submerged growth of the organism resulted in low activity even after a long period of incubation.

It was demonstrated that shaking the culture at daily intervals to encourage the formation of new pellicles did not increase total amylase production. Replacement of the medium below a pellicle with fresh medium at daily intervals did not increase the total amount of amylase produced, but it did increase the rate at which the maximum level was obtained.

Qualitative and Quantitative Nature of Bacterial Amylases.—Kneen and Beckord[1] studied the quantity and quality of amylase produced by 43 amylase-producing cultures of the *B. subtilis* group, 7 cultures of *B polymyxa*, and three cultures of *B. macerans*. The medium used for the growth of the cultures of *B. subtilis* was essentially a bran extract-

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[1] KNEEN, E., and L. D. BECKORD, *Arch. Biochem.*, 10 (No. 1), 41 (1946).

peptone-phosphate medium which was prepared as follows: Bran and distilled water were mixed in the ratio of 1 part of the former to 20 parts of the latter (by weight), autoclaved at 15 lb. pressure for 20 min., and strained through cheesecloth. To each liter of the bran extract were added 10 g. of Bacto peptone, 0.7 g. of $K_2HPO_4 \cdot 3H_2O$, and 0.3 g. of KH_2PO_4. After the constituents were dissolved, 200-ml. portions were dispensed into glass culture containers (to produce a layer about 3 cm. deep) and autoclaved at 15 lb. pressure for 20 min. The medium used for the growth of *B. polymyxa* and *B. macerans* was identical to the foregoing, except that 20 g. per liter of $CaCO_3$ were added for the purpose of neutralizing the acids produced during the growth of these organisms. A solid medium was prepared by adding 1.5 per cent of agar to the bran extract-peptone-phosphate broth.

The inoculum for a 200-ml. portion of the above-described media was prepared by growing the organism on a slant of the solid medium for 24 hr. at 35°C. and suspending the growth in 5 ml. of sterile water.

The bacteria were grown for 4 days at 35°C. for the production of the maximum amylase content, according to Kneen and Beckord.[1]

Evaluation of dextrinizing and saccharifying activities were carried out as outlined on page 530.

Classification.—On the basis of their starch dextrinizing and saccharification actions, Kneen and Beckord[2] classified the amylase-producing organisms that they studied into four groups. These were as follows:

Group 1. *B. subtilis* (saccharifying type). The production of amylase varies from little or none to relatively large amounts. During the period of starch dextrinization, but little saccharification occurs; however, saccharification is pronounced during the postdextrinization period. The level of the conversion of starch to sugar may be high. An inhibitor present in wheat retards the action of the amylase. The bacteria of this group may be isolated from plant materials.

Group 2. *B. subtilis* (nonsaccharifying or α-amylase type). Large quantities of characteristic α-amylase are produced by organisms of this group. The enzyme seems to be much like the commercial type of bacterial amylase. Bacteria producing this type of amylase may be isolated from ropy bread.

Group 3. *B. polymyxa*. The amylase or amylase system possesses starch-degrading abilities like those of an extract of barley malt. The level of fermentable sugar production is high during both the dextrini-

[1] Kneen, E., and L. D. Beckord, *Arch. Biochem.*, **10** (No. 1): 41 (1946).
[2] *Ibid.*

zation and postdextrinization periods and conversion comparable to that produced by malt is obtained.

Group 4. B. macerans. The starch is converted initially to "Schardinger dextrins," which are nonreducing and nonfermentable, but there follows a progressive production of fermentable sugars. High levels of conversion may be obtained as a result of using high enzyme concentrations or sufficient time.

Evaluation of Dextrinizing Activity.—The dextrinizing activity—the time in minutes required by the enzyme to hydrolyze 20 ml. of 1 per cent gelatinized starch to the point where a red-brown color is produced with iodine—was carried out by Kneen and Beckord,[1] using a modification of the Wohlgemuth[2] method. In carrying out the test, a 20-ml. portion of 1 per cent boiled soluble starch is buffered to a pH of 6.0 with phosphates (3.0 g. of KH_2PO_4 and 0.6 g. of $K_2HPO_4 \cdot 3H_2O$ per liter). In their studies concerned with the production of amylases on wheat bran, Beckord, Kneen, and Lewis[3] added a 10-ml. aliquot of a 1:50 extract (50 ml. of extractant per 1 g. of dry bran) of the bacterial bran to the 20 ml. of soluble starch. In their studies with liquid substrates, Kneen and Beckord added 10 ml. of enzyme preparation to the soluble starch of which 0.5 to 10 ml. was enzyme-containing substrate. *The conversion temperature was* 30°C.

Evaluation of Starch-saccharifying Activity.—The following procedure was used by Kneen and Beckord[1] for evaluating the saccharification activities of amylase preparations: In brief, it consists of measuring the carbon dioxide produced during the yeast fermentation of the hydrolytic products resulting from the action of the amylase on soluble starch. The conversions, fermentations, and gas measurements are carried out in a

.... 0.6 g. of
.   .   .   .   . of
$NaCl$ 0.04 g. of $Mg. SO_4 \cdot 7H_2O$, 0.04 g. of KH_2PO_4 0.011 g. or ....)-
$3H_2O$, and 0.03 g. of dextrose (used to accelerate the fermentation) are added to each of the cups of the pressure-meter. The amylase-containing extract together with enough distilled water to make a total volume of 10 ml. are added to the 15 ml. of nutrient starch solution described above. The mixture is incubated at 30°C. for 1 hr. Then 5 ml. of a suspension prepared to contain 0 5 g. of compressed bakers' yeast, 0.00008 g. of thiamin, and 0.00008 g. of pyridoxine are added, the top of the pressure-

[1] *Ibid.*

[2] WOHLGEMUTH, J., *Biochem. Zeit.*, **9**: 1 (1908).

[3] BECKORD, KNEEN, and LEWIS, *loc. cit.*

[4] SANDSTEDT, R M., and M. J. BLISH, *Cereal Chem.*, **11**: 368 (1934).

meter is affixed, and incubation carried out at 30°C. The pressure is equalized at the end of 5 min.

Alpha-amylase.—The alpha-amylase produced by *B. subtilis* has an optimum pH of 6.5 to 8,[1] is active at relatively high temperatures, 75 to 80°C. (even up to 95°C.), and may resist a short boiling in the presence of starch when the pH of the medium is 7 to 8. Alpha-amylase is the main amylase of bacteria. This enzyme is dextrinogenic

Uses of Bacterial Enzymes.[1,2]—The amylases and proteases produced by bacteria may be used for the same general purposes as those produced by molds (see Chap XXXIII). Bacterial amylases may be used for the desizing of textiles; in the preparation of sizes for paper; for the production of starch-conversion products of low fermentability, which are occasionally used in the brewing industry; in the liquefaction of unmalted cereals; in the clarification of beer; in the preparation of chocolate sirups, in which case the chocolate starch is dextrinized and thus the sirup does not become thick; and for other purposes.

Bacterial proteases may be used for the unhairing and bating of hides; for the degumming of silk; for desizing acetate rayon when the size is made of gelatin or casein, for the separation of silver from photographic films by digestion of the gelatin of the film and liberation of the silver salts; and for other purposes

RIBOFLAVIN PRODUCTION

The production of riboflavin (vitamin G) by bacteria of the acetone-butanol group has been described by Yamasaki,[3] by Meade, Pollard, and Rodgers,[4] by Leviton,[5] and by others.

Briefly, the process involves the selection, preparation, and sterilization of a suitable carbohydrate-containing mash; the addition of a buffer, such as CaCO₃, prior to or during the fermentation, the inoculation of the mash with a pure culture of *Clostridium acetobutylicum* or related organism, and incubation at 37 to 40°C. for 48 to 72 hr.

A fairly large number of raw materials may be used for the fermentation. Yamasaki,[3] for example, mentions in his patent the use of mashes prepared from barley, corn (maize), millet, oats, rye, and sorghum (these

[1] WALLERSTEIN, *loc cit*

[2] WILLIAMS, J J, Abstracts of Communications, Third International Congress of Microbiology, p 335, New York, Sept 2 9, 1939

[3] YAMASAKI, I , U S Patent 2,297,671, Sept 29, 1942

[4] MEADE, R L , H L POLLARD, and N E ROBBINS, U S Patent 2,369,680, Feb 20, 1945

[5] LEVITON, A , *Jour. Am. Chem. Soc*, **68**: 835 810 (1946)

TABLE 117.—PENTONIC ACIDS AND *Pseudomonas* SPECIES WHICH PRODUCE THEM[1]

d-Arabonic acid[2]	l-Arabonic acid[3]	d-Xylonic acid[4]	d-Ribonic acid[5]
Ps. fragi	Ps. fluorescens	Ps. fluorescens	Ps. fluorescens
Ps. graveolens	Ps. fragi	Ps. fragi	Ps. fragi
Ps. synxantha	Ps. mildenbergi	Ps. graveolens	Ps. graveolens
Ps. vendrelli	Ps. putida	Ps. mildenbergi	Ps. mildenbergi
	Ps. synxantha	Ps. ovalis	Ps. ovalis
	Ps. vendrelli	Ps. putida	Ps. putrifaciens
			Ps. synxantha
			Ps. vendrelli

[1] Prepared from data presented in article by L. B. Lockwood and G. E. N. Nelson, *Jour. Bact.*, 52 (No. 5), 581 (1946).

[2] Medium contained 4.8 g. of d-arabinose per 100 ml.

[3] . . . . per 100 ml.

[4] . . . . 100 ml.

[5] . . . . 100 ml.

Pseudomonas were found to oxidize lactose and maltose, but their rate of oxidation was too slow to be of interest.

Inoculums were prepared as follows. A 24-hr. culture of *Ps. graveolens* (or other species), grown in 8 ml. of a liver extract medium containing 0.2 per cent of glucose, was used to inoculate 100 ml. of fermentation solution in a Pyrex tube fitted with finely porous stones for aeration purposes. Each tube also received 1 ml. of a sterile (20 per cent) urea solution and 2.5 g. of sterilized, dry $CaCO_3$ at this time. The tubes were aerated with 100 ml. of air per minute while being incubated at 30°C.

The fermentation medium contained per liter about 100 g. of the sugar, 0.6 g. of KH_2PO_4, 0.25 g. of $MgSO_4 \cdot 7H_2O$, and 5 ml. of corn-steep liquor.

In carrying out fermentations with the rotary fermenters, 100 ml. of inoculum, prepared as described above, was used to seed 3,000 ml. of the sterile fermentation medium in a drum. During the fermentation, the drum received 1,200 ml. of air per minute, while being rotated at the rate of 9.5 r.p.m. The temperature was maintained at 25°C.

At the completion of fermentation, 100-ml. portions of the culture liquor were filtered and dried by lyophilization and analyzed.

FERMENTATION OF CELLULOSE

A knowledge of cellulose fermentation is especially important to the soil microbiologist and to those interested in the microbiology of textiles (Chap. XXXVII) and wood (Chap. XXXVIII). In view of the occurrence of such large quantities of cellulose in nature, and especially in the plant foods of animals, it is somewhat surprising to find that *the enzyme*

"cellulase" appears to be absent in herbivores and other mammals. However, cellulose-fermenting bacteria are invariably found in the intestines of herbivores, and even, occasionally, in the intestines of human beings. Some types of cellulose can thus be made available as food for animals having a vegetarian diet.

Cellulose fermenters may be divided into two main groups, the aerobic organisms, including both bacteria and fungi, and the anaerobic or microaerophilic bacteria, which usually include the types found in the intestines. Bacterial types capable of attacking cellulose belong to six principal genera; *Cellulomonas*, small Gram-negative, nonsporeforming rods; *Cytophaga*, long flexuous rods; *Cellvibrio*, *Cellfalcicule*, *Clostridium*, and *Actinomyces*.

Cellulose-fermenting organisms are also found in the soil, in some surface waters, especially in the deposited material at mouths of slow streams, in fermenting manure, in compost heaps, in decaying sawdust, etc.

The breakdown of cellulose to glucose is the result of two enzymes: cellulase and cellobiase. Cellulase converts cellulose to cellobiose, while cellobiase converts cellobiose to glucose. This breakdown may be illustrated by the following scheme [1]
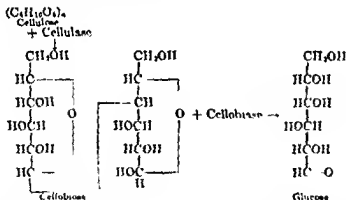
The end products formed depend on the organisms and the conditions of the fermentation. Omeliansky worked many years ago with two sporeforming anaerobes. One of these produced principally fatty acids, carbon dioxide, and methane from cellulose, while the second produced fatty acids, carbon dioxide, and hydrogen. Khouvine reported the production of acetic and butyric acids, ethyl alcohol, carbon dioxide, and hydrogen by *B. cellulose dissovens* (*Cl. dissolvens*). This fermentation

[1] Salle, A. J., "Fundamental Principles of Bacteriology," 3d ed., McGraw-Hill Book Company, Inc., New York, 1948.

is a stage in the natural process by which methane is produced in muds and silt and in sewage sludges from cellulose.

Virtanen[1] has demonstrated that much of the cellulose of finely divided wood may be fermented by enriched cultures of thermophilic cellulose-fermenting bacteria. For example, 33.9 per cent of the cellulose found in birch dust was fermented in 10 to 14 days at 61°C. The amount of cellulose fermented is proportional to the fineness of the wood dust. Virtanen is of the opinion that cellulose is not chemically bound with lignin in wood.

Considerable research has been undertaken by Boruff, Buswell, Levine, and others concerning the utilization of cellulosic wastes for the production of fuel gas. Cellulose fermenters are important in these fermentations, which are anaerobic in nature.

For a further study of the fermentation of cellulose, it is suggested that the reader refer to some of the publications cited at the end of the chapter.

SILAGE

Silage (ensilage) is the fermented product made in silos for cattle food. Although it is most commonly prepared from corn, various other substances, such as sorghum, sunflowers, clover, alfalfa, peas, soybeans, oats, rye, and wheat may be used successfully, provided certain precautions are observed. Forty to sixty pounds of molasses per ton of silage, or green corn, is usually mixed with most of these plants, excepting sorghum and sunflowers.

Procedure.—Corn is harvested and fed into a machine that cuts it into small pieces and blows them to the top of the silo. The inlet at the top of the silo may or may not contain a device for distributing the corn evenly around the silo. The corn is spread uniformly and tramped by men. A fermentation, which is primarily a lactic acid fermentation, ensues.

Harvesting the Corn.—Corn should be harvested[2] when it contains approximately 30 per cent of dry matter. It is usually several weeks after the corn has reached its maximum fresh weight before it contains this amount of dry matter. During the latter part of the growing season, the increase in dry matter occurs principally in the ears.

If the corn contains over 30 per cent of dry matter, it does not usually keep as well in the silo, for, owing to difficulties in cutting it finely, in distributing it in the silo, and in packing it, air spaces are likely to appear.

[1] VIRTANEN, A I , Abstracts of Communications, *Third International Congress* of Microbiology, p 333, New York, Sept. 2-9, 1939

[2] NEVENS, W B , *Ill. Agr. Expt. Sta Circ.*, 463, November, 1936

Molds frequently grow in these air spaces, spoiling the product. On the other hand when the dry matter content is much less than 30 per cent, there is usually a loss in the future feeding value of the product.

Packing the Silage.—Silage is packed to exclude the air, which favors mold growth and subsequent spoilage.

If the corn contains more than approximately 35 per cent of dry matter, water should be added to aid in packing it more closely. The incoming corn should be spread evenly around the silo by means of a special distributor as it enters, since ordinarily there is a tendency for the dry, light portions of the corn to accumulate at a point which is farthest away from the corn inlet and since it is difficult to pack such corn without leaving air pockets. It is important, likewise, to guard against uneven settling of the corn in the silo. Accordingly, at least two men should tramp the corn.

When the corn contains less than 35 per cent of dry matter, packing is not a special problem and it is unnecessary to add water. A higher acidity develops in silage prepared from immature corn.

Fermentation.—During the fermentation that ensues, acids and alcohols are produced, as well as esters. At least 1 per cent of acid is formed, lactic acid being the main acid. Acetic, propionic, butyric, and sometimes formic acids are produced. Besides small amounts of ethyl alcohol, traces of the higher alcohols may be found.

Bacteria, yeasts, and molds may be found in the ensilage. Normal fermentation is brought about by the lactobacilli, especially *Lactobacillus bulgaricus*, while certain other bacteria produce lactic acid in small amounts. The acid formed during fermentation inhibits the development of those types of bacteria which normally bring about putrefactive changes. Members of the colon-aerogenes group of bacteria occasionally *produce gas in silage.* Yeast-like organisms are found during the early part of the fermentation—they are usually members of the mycoderma group. Molds develop only in the presence of oxygen. Consequently the spoilage of much silage by molds is unnecessary. When it occurs it usually indicates a lack of proper care in packing or covering, or a defective silo.

In some instances, silage has caused serious poisoning when fed to livestock. It has been shown that *Clostridium botulinum* is usually the cause of such forage poisoning.

Lack of sufficient sugar for the proper growth of the lactic acid bacteria, or the development of thermophilic bacteria, in silage sometimes leads to the production of so-called "sweet silage."

Use of Molasses in Grass Silage.—The subject of the use of molasses in grass silage preparation has been reviewed by Bender (1948)

MICROBIOLOGY OF LEATHER MANUFACTURE

The essential steps in *preparing fresh skins and hides for leather* include preservation, soaking and fleshing, unhairing, "scudding," "bating," "drenching," pickling, and finally tanning and finishing. Obviously, there are many methods and modifications used. In several of these processes microbiological activities are implicit. The subject will be discussed very briefly.

Skins and hides as removed from animals are richly supplied with microbes, which if unchecked would rapidly injure or destroy the tissues. However, this activity can be largely prevented and the leather-making material may be preserved by salting (curing), by drying, by combining drying and salting, by pickling, or by use of disinfectants. The first of these methods is most commonly practiced.

Soaking is carried out to remove blood, dirt and manure, salt, and other soluble material; and at the same time to "plump," or swell, and to soften the skins and hides by absorption of water. During soaking there is danger of undesirable bacterial action unless controlled. By keeping the soak water cool (*i.e.*, below optimum temperature) and by changing it several times, bacterial growth may be somewhat inhibited.

In the so-called "fleshing" of hides, adipose tissue and other undesirable portions are trimmed from the hides.

Various methods are employed for unhairing the skin or hide. In the oldest method, the hides were placed in a sweating chamber, where the humidity was high and the atmosphere warm. Bacteria inherent on the surface, especially   *. . . and dis-*
solution of some of :
This process was commonly employed with sheepskins, and the hence could then be "pulled" in intact form. Sweating processes could be controlled only with difficulty, and consequently the hides or skins were injured, many of them severely. Lime pits or saturated lime water baths have been more commonly used in unhairing processes, the skins being swelled and hair sheaths dissolved at a pH of about 12 5, according to Wilson.

Limewater containing sodium or potassium sulphide or other depilatory compounds, a sodium sulphide and lime paste, alkalies, acids or enzymes, which may include pancreatin solutions, or bacterial or mold proteases, have been used at various periods in unhairing processes. The most recent trend is toward the use of enzymes.

The loosened hair, sebaceous and sudoriferous glands, dirt and the lime soaps formed, and other material (not a part of the true skin) are removed by scudding, or scraping with a tool comparable to a large

knife or drawshave with a smooth but dull edge. The skins or hides that have been limed contain lime salts that must be removed It is also important to remove all other material except the collagen fibers that make up the firm tissues of the different layers of the skin This removal is accomplished by bating

Bating is an enzymic process for bringing about physical and chemical changes in the hides or skins, in which neutral salts may or may not be used The oldest methods consisted in using infusions of dog manure, known as "puring," or of pigeon or fowl manure, known as "bating." It was shown by Wood that the effective action of the dung was due to the enzymes contained in it—lipase, rennet, and peptic, tryptic, and amylolytic enzymes Later pancreatic enzymes or other enzyme mixtures were substituted for the dung bates. In this process, the pH is usually slightly on the alkaline side of neutrality.

During bating, the enzymes cause the removal of the coagulated or coagulable proteins of the hide, the dissolved keratin, and reticular tissue. The skins are partially delimed, grain roughness may be removed to some extent, and the stretching ability of the elastin fibers of the skin is restored [1] The last three of these changes may be brought about by the use of lactic acid or ammonium chloride. The pH is likewise adjusted during bating.

The skins of sheep, goats, and calves are sometimes "drenched" in a bran infusion. Bacteria ferment the carbohydrates of the bran with the production of organic acids, especially lactic, and gases. Drenching is effective because the lactic acid forms easily soluble lime salts that can be washed out and thus prepares the skins for the eventual process of tanning The hides may be pickled in a bath containing dilute sulphuric acid and sodium chloride previous to tanning.

CURING OF TOBACCO

Tobacco undergoes a series of curing treatments before it is manufactured, in order to improve its texture and aroma Whether these changes are to any marked extent the results of specific bacterial fermentation has been a subject of discussion for many years. It is now generally believed, as a result of the evidence accumulated over many years, that the principal changes taking place during the curing process are largely the result of the action of the enzymes, especially the oxidases, present in the tobacco leaves and not dependent on bacterial action. Nevertheless, the changes induced by the large number of bacteria present

[1] McLaughlin, G. D., J. H. Highberger, F. O'Flaherty, and K. Moore, *Jour. Am Leather Chem Assoc*, **24**: 339–379 (1929)

might easily serve as a contributory factor in the whole process. A few earlier studies indicated that the finer qualities of tobacco were the results of the action of specific types of bacteria. This seems never to have been confirmed by carefully controlled and adequate research.

After harvesting, the tobacco leaves are dried, piled in heaps, moistened (sometimes with dilute solutions of sugars, malt extract, honey, or other sirups) and permitted to undergo spontaneous fermentation or curing. During this process heat is evolved and the temperature of the mass may rise to 55 to 60°C. The physical appearance and chemical structure of components of the leaves are changed. Aroma and flavor are developed. Starch and reducing sugars tend to disappear. The quantities of malic acid, nicotine, pentosans, and protein decrease, while there is an increase in the quantity of citric acid. Carbon dioxide and ammonia are evolved. Hydrolytic, oxidative, proteolytic, and other types of enzymes are apparently concerned in the curing process.

As would be expected from the mode of cultivation the flora on the leaves may be quite varied. According to a summary of the many bacterial studies prepared by Salle,[1] Bacillus subtilis, B. mycoides, B polymyxa, Proteus vulgaris, and species of the genus Aspergillus and other molds are present on tobacco leaves. This list is probably incomplete. Giovannozzi states that the unfermented leaf of Kentucky tobacco may contain from 100,000 to 100,000,000 bacteria per gram (dry weight) Blastomycetes also may develop during the first part of the fermentation, but may disappear later. Cocci are likewise present in fermented tobacco, frequently in larger numbers than the bacilli.[2]

This fermentation, like that of silage, grass, and other materials in which components of living leaf tissues and the adherent microbes may both function, will continue to offer an inviting field for microbiological research.

ETHANOL PRODUCTION BY BACTERIA

Ethanol is produced in varying quantities by a relatively large number of bacteria. Usually the ethanol is produced in small amounts only and is one of several products, thus making industrial production unfeasible. Research may somewhat change this viewpoint, but bacteria are not likely to be serious competitors of yeast in the near future.

A medium containing 2 per cent glucose and yeast extract was fermented by Termobacterium mobile Lindner (Lactobacillus mobile) with the production of 45.2 per cent of ethanol, 45 1 per cent of carbon dioxide,

[1] SALLE, loc. cit.
[2] GIOVANNOZZI, M , Chimica e industria (Italy), 40: 768 (1937)   (Chem. Abstract)

and 7.2 per cent of lactic acid.[1] A fermentation using this organsim has been carried out on an industrial scale in Germany. The organism was isolated in Mexico by Lindner from the fermenting juice of a large cactus. This fermented juice, locally used as an alcoholic beverage, is called "pulque."

Weizmann[2] has reported a yield of 25 6 per cent of ethanol from a sucrose solution by *Clostridum ethylicum* (Weizmann). From 720 g. of cane sugar, 24 g. of volatile acids, as butyric acid, were formed The organism was isolated from *Hibiscus sabdariffa*.

Bacillus asiaticus mobilis Castellani, an organism believed to be closely related to *Escherichia coli*, gives rise to ethanol, hydrogen, and butylene glycol.

Sarcina ventriculi[3] Goodsir forms ethanol, carbon dioxide, and acetic acid as the principal products from glucose In one experiment reported by Kluyver, 43 7 per cent of the glucose was fermented to ethanol. *Sar. ventriculi* is aerobic[4] and has an optimum temperature of 30 to 35°C.

Ethanol is produced by certain acetic acid bacteria, for example, *Acetobacter ascendens*, *A. suboxydans*, and *A. pasteurianum*, under anaerobie conditions.[5]

It is produced in small quantities by heterofermentative lactic acid bacteria, by certain cellulose-fermenting bacteria, and by the bacteria that produce butanol and acetone.

ACETYLMETHYLCARBINOL, 2,3-BUTYLENE GLYCOL, AND DIACETYL

Acetylmethylcarbinol and 2,3-butylene glycol are frequently found together in certain fermentations Refer to Chap. XXII for a consideration of the 2,3-butylene glycol fermentation.

Some bacteria have the ability to reduce acetylmethylcarbinol to 2,3-butylene glycol, a change that may be indicated as follows:

$$
\begin{array}{ccc}
CH_3 & & CH_3 \\
| & & | \\
C=O & & CHOH \\
| & +2H \rightarrow & | \\
CHOH & & CHOH \\
| & & | \\
CH_3 & & CH_3 \\
\end{array}
$$

Acetylmethylcarbinol   2 3 Butylene glycol
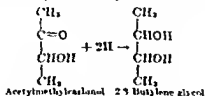
[1] LINDNER, P, "Mikroskopische und biologische Betriebskontrolle in den Gärungsgewerben, ." Paul Parey, Berlin, 1930
[2] WEIZMANN, C, *Jour. Soc Chem Ind.*, 57: 1049 (1938)
[3] SUIT, J, "Die Gärungssreinen," Gustav Fischer, Jena, 1930, KLUYVER, A J., "The Chemical Activities of Micro-organisms," University of London Press, Ltd, London, 1931
[4] KLUYVER, loc. cit
[5] BUTLIN, K R., "The Biochemical Activities of the Acetic Acid Bacteria," Chemistry Research, Special Report 2. H M. Stationery Office, London, 1936

Other bacteria are capable of oxidizing 2,3-butylene glycol to acetylmethylcarbinol. One such organism is *A. acrogenes*. Still other bacteria may oxidize acetylmethylcarbinol to diacetyl:

$$\begin{array}{ccc}
CH_3 & & CH_3 \\
| & & | \\
C=O & & C=O \\
| & + \tfrac{1}{2}O_2 \rightarrow & | & + H_2O \\
CHOH & & C=O \\
| & & | \\
CH_3 & & CH_3 \\
\text{Acetylmethylcarbinol} & & \text{Diacetyl}
\end{array}$$
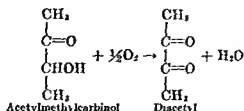
Diacetyl is important as an aroma-producing component of butter. Butter cultures of the desired type always contain diacetyl and acetylmethylcarbinol in relatively large amounts. The latter substance is oxidized to diacetyl in butter cultures by such citric acid fermenting organisms as *Streptococcus citrovorus* and *Strept. paracitrovorus*, according to Michaelian and Hammer.[1] The production of acetylmethylcarbinol and especially diacetyl by butter cultures or pure cultures of the citric-acid-fermenting streptococci is favored by the acidification of the cultures with a mixture of citric and sulphuric acids and the presence of an abundant supply of oxygen. Obviously the reductions of diacetyl and acetylmethylcarbinol to 2,3-butylene glycol cause a diminution in the amount of the aroma-producing substance, diacetyl.

FERMENTATION OF RHAMNOSE
BY BACTERIUM RHAMNOSIFERMENTANS[2]

The fermentation of rhamnose ($CH_3 \cdot CHOH \cdot CHOH \cdot CHOH \cdot CHOH \cdot CHO$) by *Bact. rhamnosifermentans* is of particular interest, because propylene glycol ($CH_3 \cdot CHOH \cdot CH_2OH$) is formed as one of the end products.

The organism was described by Castellani[3] as *Bacillus rhamnosifermentans*. It is a Gram-negative, facultative aerobe, which shows selective action in respect to the carbohydrates fermented and the nitrogen compounds utilized. Rhamnose in peptone water (1 per cent) containing 1 5 per cent of calcium carbonate was fermented nearly completely in 10 days by *Bact. rhamnosifermentans*, and more vigorously than any other carbohydrate investigated.[4] Yeast water was unfavor-

[1] MICHAELIAN, M. B., and B W. HAMMER, *Iowa Agr. Expt Sta , Research Bull.* 205, 1936
[2] KLUYVER, A J., und CH. SCHNELLEN, *Enzymologia,* 4: 7–12 (1937).
[3] CASTELLANI, A , *Ann. Inst. Pasteur,* 47: 297–305 (1931)
[4] KLUYVER und SCHNELLEN, *loc cit.*

able as a nitrogen source. Equimolar quantities of hydrogen and carbon dioxide gases were produced by *Bact. rhamnosifermentans*, which is believed to be closely related to *Escherichia coli*.

Table 118 shows the products identified and quantitatively estimated in the fermentation of a medium, which contained 1.5 per cent rhamnose and 1.5 per cent calcium carbonate in peptone water. The amount of propylene glycol represented was obtained by calculation and not by quantitative estimation of its content in the fermentation medium.

On the basis of the available facts and the assumption concerning propylene glycol, Kluyver and Schnellen have suggested the following scheme to indicate the course of the fermentation of rhamnose by *Bact. rhamnosifermentans*:
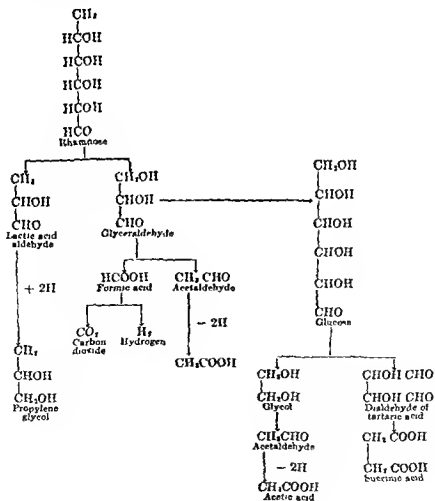
TABLE 118.—QUANTITATIVE RESULTS OF THE FERMENTATION OF RHAMNOSE BY *Bact rhamnosifermentans*[1]

	Weight, grams	Per cent carbon	Milli-mols	Millimols per 100 millimols of fermented rhamnose	Milliequivalents of available hydrogen	Available hydrogen, per cent
Rhamnose:						
Added	9 956					
Recovered . . .	2 737					
Fermented	7 210	100	44	100	2,600	100
Carbon dioxide	0 623	5 36	14	31.8		
Hydrogen . .	0 032		14	31 8	63.6	2 4
Formic acid	0.057	0 50	1	2 3	4.6	0 2
Acetic acid	1 935	24 42	32	73 0	584 0	22.5
Ethanol	0 056	1 00	1	2 3	27 6	1.1
Succinic acid	1 752	22 50	15	34.0	476.0	18 3
Propylene glycol	3 344*	50 00	44	100	1,600	61 0
Total		103 78			. . . . .	105.5

* Not determined, but computed
[1] KLUYVER, A J., und CH SCHNELLEN, *Enzymologia*, 4: 7–12 (1937).

References on Minor Fermentations
GENERAL

BARKER, H A.: On the Biochemistry of the Methane Fermentation, *Arch. Mikrobiol*, 7: 404–419 (1936).

———: The Production of Caproic and Butyric Acids by the Methane Fermentation of Ethyl Alcohol, *Arch Mikrobiol*, 8: 415–421 (1937).

———: On the Fermentation of Glutamic Acid, *Enzymologia*, 2: 175–182 (1937)

BUTLIN, K R : "The Biochemical Activities of the Acetic Acid Bacteria," Chemistry Research, Special Report 2, H. M. Stationery Office, London, 1936

CASTELLANI, A.: Sur un bacille déterminant la fermentation du rhamnose avec dégagement de gaz, à l'exclusion de tout autre hydrate de carbone, glucoside, alcool, ou composé hydro-aromatique, d'une série de 28 de ces substances examinées Emploi de ce bacille pour découvrir et identifier le rhamnose, *Ann Inst Pasteur*, 47: 297–305 (1931)

DONKER, H. J. L. : "Bijdrage tot de kennis der boterzuurbutyl-alcoholen acetongistungen," Thesis, Technische Hoogeschool, Delft, 1926

EVANS, T H, and H HIBBERT: Bacterial Polysaccharides, *Scientific Report Series No* 6, Sugar Research Foundation, Inc., New York, April, 1947.

GIOVANNOZZI, M.: *Chimica e industria* (Italy), 40: 768: 1937.

KLUYVER, A J.. "The Chemical Activities of Micro-organisms," University of London Press, Ltd., London, 1931.

———: Microbial Metabolism and Its Significance to the Microbiologist, Report of Proc Third International Congress of Microbiology, New York, 1940

——— und CH SCHNELLEN: Über die Vergärung von Rhamnose, *Enzymologia*, 4: 7–12 (1937).

——— and ———: On the Fermentation of Carbon Monoxide by Pure Cultures of Methane Bacteria, *Arch. Biochem*, **14** (Nos 1 & 2): 57–70 (1947).

LANGLYKKE, A. J., W. H PETERSON, and E McCOY. Products from the Fermentation of Glucose and Arabinose by Butyric Acid Anaerobes, *Jour. Bact.*, 29: 333 (1935).

LINDNER, P.. "Mikroskopische und biologische Betriebskontrolle in den Gärungsgewerben," 6th ed , p 585, Paul Parey, Berlin, 1930.

SUIT, J.: "Die Gärungssarcinen," Gustav Fischer, Jena, 1930

WALLERSTEIN, L : Enzyme Preparations from Microorganisms   Commercial Production and Industrial Application, *Ind Eng Chem*, **31**: 1218 (1939)

WEIZMANN, C· Preliminary Note on an Ethyl Alcohol Producing Bacillus Isolated from *Hibiscus sabdariffa* L. (Rozella), *Jour Soc Chem Ind*, **57**: 1019 (1938).

WILLAMAN, J J . Industrial Use of Microbial Enzymes, Abstracts of Communications, Third International Congress of Microbiology, pp 335–336, New York, Sept. 2–9, 1939

THE CELLULOSE FERMENTATIONS

"Bergey's Manual of Determinative Bacteriology," 6th ed , The Williams & Wilkins Company, Baltimore, 1948

BERNHAUER, K "Gärungschemisches Praktikum," Verlag Julius Springer, Berlin, 1939

BORUFF, C. S, and A M BUSWELL Fermentation Products of Cellulose, Ill. State Water Survey, *Circ* 7, Urbana, 1929

BUSWELL, A M , and W D HATFIELD Anaerobic Fermentations, *Ill State Water Survey*, Bull 32, Urbana, Ill , 1939

DUBOS, R J   Cellulose Decomposition by Aerobic Bacteria, *Jour Bact*, **15**: 223 (1928).

NORMAN, A O . "The Biochemistry of Cellulose, the Polyuronides, Lignin, etc ," Oxford University Press, New York, 1937

PETERSON, W H , and S SZIESZKO   Further Studies on the Thermophilic Fermentation of Cellulose and Cellulose Materials, *Centr Bakt Parasitenk* , Abt II, **88**: 410–417 (1933)

SALLE, A J   "Fundamental Principles of Bacteriology," 3d ed , McGraw-Hill Book Company, Inc , New York, 1948

STEPHENSON, M : "Bacterial Metabolism," Longmans, Green & Company, New York, 1939

THAYSEN, A C , and H J BUNKER· "The Microbiology of Cellulose, Hemicelluloses, Pectin and Gums," Oxford University Press, New York, 1927

VIRTANEN, A I . The Fermentation of the Native Cellulose in Wood, p 333, Abstracts of Communications, Third International Congress of Microbiology, New York, Sept 2–9, 1939

WAKSMAN, S A · "Principles of Soil Microbiology," The Williams & Wilkins Company, Baltimore, 1927

——— The Microbiology of Cellulose Decomposition and Some Economic Problems Involved, *Botan Rev*, **6** (No. 12): 637–665 (1940).

SOME PATENTS

CHRISTENSEN, L M , and W. N. McCUTCHAN: Process for Producing Acids by Fermentation of Cellulosic Materials, U   Patent 1,875,368, Sept 6, 1932

HUTCHINSON, H B : Production of Alip   by Fermentation, U.S Patent 1,867,916, July 19, 1932

LANGWELL, H.: Fermentation of Cellulose, U.S. Patents 1,443,881, Jan. 30, 1923; 1,639,571, Aug 16, 1927.

————: Fermentation of Cellulosic Materials, U.S. Patents 1,602,306, Oct. 5, 1926, 1,864,838 and 1,864,839, June 28, 1932.

LEGG, D. A., and L. M. CHRISTENSEN: Process for the Production of Organic Acids from Cellulosic Material, U.S. Patent 1,864,746, June 28, 1932.

Leather

LOCHHEAD, A. G.: Bacteriological Studies on the Red Discoloration of Salted Hides, *Can. Jour. Research*, **10**: 275–286 (1934).

McLAUGHLIN, G. D., J H HIGHBENGER, F. O'FLAHERTY, and E. K. MOORE: Some Studies of the Science and Practice of Bating, *Jour. Am. Leather Chem. Assoc,* **24**: 339–379 (1929)

STUART, L S.: The Morphology of Bacteria Causing Reddening of Salted Hides, *Jour. Am Leather Chem. Assoc.*, **30**: 226–235 (1935).

————, R W. FREY, and L. H. JAMES: Microbiological Studies of Salt in Relation to the Reddening of Salted Hides, *U.S. Dept. Agr*, *Tech. Bull.* 383, Sept., 1933

WILSON, J A : "Chemistry of Leather Manufacture," 2d ed., 2 vols, Reinhold Publishing Corporation, New York, 1939.

ACETYLMETHYLCARBINOL, BUTYLENE GLYCOL, AND DIACETYL

HAMMER, B. W , G L. STAHLY, C. H WERKMAN, and M. B. MICHAELIAN: Reduction of Acetylmethylcarbinol and Diacetyl to 2,3-Butylene Glycol by the Citric Acid Fermenting Streptococci of Butter Cultures, *Iowa Agr. Expt Sta. Research Bull*, **191**: 381–407 (1935).

MICHAELIAN, M B , R. S FARMER, and B W. HAMMER The Relationship of Acetylmethylcarbinol and Diacetyl to Butter Cultures, *Iowa Agr. Expt Sta. Research Bull.*, **166**: 322–360 (1933).

———— and B. W. HAMMER: Studies on Acetylmethylcarbinol and Diacetyl in Dairy Products, *Iowa Agr. Expt. Sta. Research Bull*, **179**: 203–227 (1935).

———— and ————: The Oxidation of Acetylmethylcarbinol to Diacetyl in Butter Cultures, *Iowa Agr. Expt Sta. Research Bull.*, **205**: 203–214 (1936)

SCHMALF   ...standteil von Lebens-
und
VAN NIE   oms, *Biochem*
   Zeit, **210**: 234–251 (1929)

References Concerning Bacterial Amylases

BECKORD, L D., E. KNEEN, and K H. LEWIS: Bacterial Amylases, Production on Wheat Bran, *Ind Eng Chem*, **37**: 692–696 (1945)

————, G L. PELTIER, and E KNEEN: Bacterial Amylases Production in Thin Stillage, *Ind Eng Chem*, **38**: 232–238 (1946).

BLISH, M J., and R. M. SANDSTEDT: Biocatalytic Activators Specific for the Yeast Fermentation of Maltose, *Jour. Biol. Chem*, **118**: 765–780 (1937).

DI C/ .... l Purification and
I .... l Groups, *Arch*
  Biochem, **16** (No 3): 343–350 (1947).

———— and ———— α-Amylase from *Bacillus subtilis*: III. Effects of Inhibitors upon Liquefaction and Dextrinization. *Arch Biochem*, **17** (No. 1): 1–2 (1948).

HAO, L. C., and J. A. JUMP: Microbial Amylase Preparations, *Ind Eng Chem.*, 37: 521–525 (1945).

HOPKINS, R. H, and D. KULKA, Some Comparisons between a Bacterial Amylase and α-Malt Amylase I The Kinetics of Starch Saccharification by the Two Enzymes, *Jour Inst Brewing*, 48: 170–174 (1912)

KLINE, L, L. R MacDonnell, and H. Lineweaver: Bacterial Proteinase from Waste Asparagus Butts, *Ind Eng Chem*, 36: 1152–1158 (1944).

KNEEN, E, and L. D Beckord. Quantity and Quality of Amylase Produced by Various Bacterial Isolates, *Arch Biochem*, 10 (No 1): 41–54 (1946)

——, R M Sandstedt, and C M Hollenbeck: The Differential Stability of the Malt Amylases—Separation of the Alpha and Beta Components, *Cereal Chem.*, 20: 399–423 (1943)

SANDSTEDT, R M, E KNEEN, and M J BLISH A Standardized Wohlgemuth Procedure for Alpha-amylase Activity, *Cereal Chem*, 16: 712–723 (1939)

TILDEN, E. B, and C S HUDSON. Conversion of Starch to Crystalline Dextrins by the Action of a New Type of Amylase separated from Cultures of *Aerobacillus macerans*, *Jour. Am Chem Soc*, 61: 2900–2902 (1939)

—— and ——. Preparation and Properties of the Amylases Produced by *Bacillus macerans* and *Bacillus polymyxa*, *Jour. Bact*, 43: 527 (1942)

References on Riboflavin

LEVITOV, A : The Microbiological Synthesis of Riboflavin—A Theory concerning Its Inhibition, *Jour Am Chem Soc*, 68: 835–840 (1946)

—— and H B. Haller The Relation between Riboflavin and Hydrogen Peroxide in the Presence of Ferrous Ion, Paper presented at 110th Meeting of the American Chemical Society, Chicago, Sept 9–13, 1946

MEADE, R L, H L POLLARD, and N E RODGERS. Process for Manufacturing a Vitamin Concentrate, U S Patent 2,369,680, Feb 20, 1945

YAMASAKI, I Process of Preparing Growth Promoting Vitamin B₂ (Vitamin G) by Fermentation, U S Patent 2,297,671, Sept 29, 1942

References on the Genera Pseudomonas and Phytomonas

AUSBERG, C L. The Formation of d-Gluconic Acid by *Bacterium saratanon* Smith, *Jour Biol Chem*, 9: 1 7 (1911)

LOCKWOOD, L B, and G E N NELSON The Oxidation of Pentoses by Pseudomonas, *Jour. Bact*, 52 (No 5): 581 586 (1946)

—— and P H STODOLA Preliminary Studies on the Production of α-Ketoglutaric Acid by *Pseudomonas fluorescens*, *Jour Biol Chem*, 164 (No 1): 81–83 (1946)

—— and ——. α-Ketoglutaric Acid by Fermentation, U S Patent 2,113,919, June 22, 1918

—— , B TABENKIN, and G E WARD. The Production of Gluconic Acid and 2-Ketogluconic Acid from Glucose by Species of *Pseudomonas* and *Phytomonas*, *Jour. Bact*, 42 (No 1): 51 61 (1941)

PERUDIANSKII, V V Formation of Gluconic Acid during the Oxidation of Glucose by Bacteria, *Microbiology* (U S S R.), 8 (No. 2): 149–159 (1939); *Chem. Abs.*, 34: 7321 (1940)

—— and M A KARBETSKII: Formation of Gluconic Acids in *Bacterium putidum* L. et N, *Microbiology* (U S S R.), 8 (No 3–4): 339–352 (1939); *Chem. Abs.*, 34: 7321 (1940)

LANGWELL, H.: Fermentation of Cellulose, U S. Patents 1,443,881, Jan. 30, 1923,
    1,639,571, Aug. 16, 1927.

————: Fermentation of Cellulosic Materials, U.S. Patents 1,602,306, Oct. 5, 1926,
    1,864,838 and 1,864,839, June 28, 1932.

LEGG, D. A., and L. M. CHRISTENSEN: Process for the Production of Organic Acids
    from Cellulosic Material, U S. Patent 1,864,746, June 28, 1932.

Leather

LOCHHEAD, A. G.: Bacteriological Studies on the Red Discoloration of Salted Hides,
    Can. Jour. Research, 10: 275–286 (1934).

McLAUGHLIN, G. D., J. H. HIGHBERGER, F. O'FLAHERTY, and E. K. MOORE: Some
    Studies of the Science and Practice of Bating, Jour. Am. Leather Chem. Assoc,
    24: 339–379 (1929)

STUART, L. S.: The Morphology of Bacteria Causing Reddening of Salted Hides,
    Jour. Am Leather Chem Assoc., 30: 226–235 (1935).

————, R W. FREY, and L R. JAMES: Microbiological Studies of Salt in Relation to
    the Reddening of Salted Hides, U.S. Dept. Agr., Tech. Bull. 383, Sept , 1933.

WILSON, J. A. "Chemistry of Leather Manufacture," 2d ed , 2 vols., Reinhold
    Publishing Corporation, New York, 1939.

ACETYLMETHYLCARBINOL, BUTYLENE GLYCOL, AND DIACETYL

HAMMER, B. W , G L. STAHLY, C H. WERKMAN, and M. B. MICHAELIAN: Reduction
    of Acetylmethylcarbinol and Diacetyl to 2,3-Butylene Glycol by the Citric
    Acid Fermenting Streptococci of Butter Cultures, Iowa Agr. Expt Sta. Research
    Bull., 191: 381–407 (1935)

MICHAELIAN, M. B., R. S FARMER, and B W. HAMMER: The Relationship of Acetyl-
    methylcarbinol and Diacetyl to Butter Cultures, Iowa Agr. Expt. Sta Research
    Bull , 155: 322–360 (1933)

———— and B. W HAMMER Studies on Acetylmethylcarbinol and Diacetyl in Dairy
    Products, Iowa Agr Expt Sta. Research Bull , 179: 203–227 (1935).

———— and ————: The Oxidation of Acetylmethylcarbinol to Diacetyl in Butter
    Cultures, Iowa Agr Expt Sta Research Bull., 205: 203–214 (1936)

SCHMALFUSS, H , und H BARTHMEYER. Diacetyl als Aromabestandteil von Lebens-
    und Genussmiteln, Biochem Zeit., 310: 330–335 (1929).

VAN NIEL, C R , A J KLUYVER, und H. G DERX: Über das Butteraroma, Biochem
    Zeit , 210: 234–251 (1929)

References Concerning Bacterial Amylases

BECKORD, L D., E KNEEN, and K H LEWIS: Bacterial Amylases, Production on
    Wheat Bran, Ind. Eng Chem , 37: 692–696 (1945)

————, G L PELTIER, and E KNEEN: Bacterial Amylases Production in Thin
    Stillage, Ind Eng. Chem , 38: 232–238 (1946)

BLISH, M J , and R. M. SANDSTEDT: Biocatalytic Activators Specific for the Yeast
    Fermentation of Maltose, Jour. Biol Chem , 118: 765–780 (1937).

DI CARLO, F J , S REDFERN: α-Amylase from Bacillus subtilis: I. Purification and
    Physical Properties, Arch. Biochem , 15 (No. 3). 333–342 (1947)

———— and ————; α-Amylase from Bacillus subtilis: II Essential Groups, Arch
    Biochem., 15 (No. 3): 343–350 (1947).

———— and ————: α-Amylase from Bacillus subtilis: III Effects of Inhibitors upon
    Liquefaction and Dextrinization Arch Biochem , 17 (No 1). 1–2 (1948)

HAO, L. C., and J. A. JUMP. Microbial Amylase Preparations, *Ind Eng. Chem.*, 37: 521–525 (1945)

HOPKINS, R. H., and D KULKA· Some Comparisons between a Bacterial Amylase and α-Malt Amylase I The Kinetics of Starch Saccharification by the Two Enzymes, *Jour Inst Brewing*, 48: 170–174 (1942)

KLINE, L, L R MACDONNELL, and H LINEWEAVER Bacterial Proteinase from Waste Asparagus Butts, *Ind Eng. Chem.*, 36: 1152–1158 (1944).

KNEEN, E, and L D BECKORD Quantity and Quality of Amylose Produced by Various Bacterial Isolates, *Arch Biochem*, 10 (No 1): 41–54 (1946)

———, R M SANDSTEDT, and C M HOLLENBECK· The Differential Stability of the Malt Amylases—Separation of the Alpha and Beta Components, *Cereal Chem.*, 20: 399–423 (1943)

SANDSTEDT, R. M, E KNEEN, and M J BLISH A Standardized Wohlgemuth Procedure for Alpha-amylase Activity, *Cereal Chem*, 16: 712–723 (1939)

TILDEN, E B, and C S HUDSON Conversion of Starch to Crystalline Dextrins by the Action of a Live Culture of Amylase Separated from Cultures of *Aerobacillus macerans*, *Jour Am Chem Soc*, 61· 2900–2902 (1939)

——— and ——— Preparation and Properties of the Amylases Produced by *Bacillus macerans* and *Bacillus palymyxa*, *Jour. Bact*, 43: 527 (1942)

References on Riboflavin

LEVITON, A · The Microbiological Synthesis of Riboflavin—A Theory concerning Its Inhibition, *Jour Am Chem Soc*, 68: 835–840 (1946)

——— and H B HALLER The Relation between Riboflavin and Hydrogen Peroxide in the Presence of Ferrous Ion, Paper presented at 110th Meeting of the American Chemical Society, Chicago, Sept 9–13, 1946

MFADL, R E, H L POLLARD, and N E RODGERS Process for Manufacturing a Vitamin Concentrate, U S Patent 2,369,680, Feb 20, 1945

YAMASAKI, I Process of Preparing Growth Promoting Vitamin B₂ (Vitamin G) by Fermentation, U S Patent 2,297,671, Sept 29, 1942

References on the Genera Pseudomonas and Phytomonas

ALSBERG, C L The Formation of d-Gluconic Acid by *Bacterium suastanos* Smith, *Jour Biol Chem*, 9: 1–7 (1911)

LOCKWOOD, L B, and G E N NELSON The Oxidation of Pentoses by Pseudomonas, *Jour Bact*, 52 (No 5): 581–586 (1946)

——— and F H STODOLA Preliminary Studies on the Production of α-Ketoglutaric Acid by *Pseudomonas fluorescens*, *Jour Biol Chem*, 164 (No 1): 81–83 (1946).

——— and ——— α-Ketoglutaric Acid by Fermentation, U.S Patent 2,443,919, June 22, 1948

———, B TABENKIN, and G E WARD The Production of Gluconic Acid and 2-Ketogluconic Acid from Glucose by Species of *Pseudomonas* and *Phytomonas*, *Jour. Bact*, 42 (No. 1): 51–61 (1941)

PERVOZVANSKII, V. V : Formation of Gluconic Acid during the Oxidation of Glucose by Bacteria, *Microbiology* (U.S.S.R.), 8 (No 2). 119–159 (1939), *Chem Abs*, 34: 7321 (1940).

——— and M. A. IVASHKEVICH: Formation of Gluconic Acids in *Bacterium putidum* L et N, *Microbiology* (U.S.S.R.), 8 (No. 3-4): 339–352 (1939); *Chem. Abs*, 34: 7321 (1940)

STANIER, R. Y.: Acetic Acid Production from Ethanol by Fluorescent Pseudomonads, *Jour. Bact.*, **54** (No. 2): 191–194 (1947).

STODOLA, F. H., and L. B. LOCKWOOD: The Oxidation of Lactose and Maltose to Bionic Acids by Pseudomonas, *Jour. Biol. Chem.*, **171** (No. 1): 213–221 (1947).

STUBBS, J. J., L. B. LOCKWOOD, E. T ROE, B. TABENKIN, and G. E. WARD: Bacterial Production of Ketogluconic Acids from Glucose, *Ind. Eng. Chem.*, **32**: 1626–1631 (1940).

VAUGHN, R. H.: The Acetic Acid Bacteria, *Wallerstein Labs. Communs.*, **5** (No 14): 5–26 (1942).

References on Silage

ALLEN, P. W.: "Industrial Fermentations," Reinhold Publishing Corporation, New York, 1926.

KRAUSS, W E., C. C HAYDEN, A. E. PERKINS, and R. G. WASHBURN: A Trial with Temporary Silos, *Ohio .1gr Expt Sta., Bimonthly Bull ,* **23**: 71 (1938).

MARSHALL, C. E. : "Microbiology," The Blakiston Company, Philadelphia, 1921.

NEVENS, W. B.: Silage Crops for Dairy Cattle, *Ill. Agr. Expt. Sta., Circ.* 463, November, 1936.

THE MOLDS

True molds are members of the division of the plant kingdom known as *Thallophyta*. They do not possess chlorophyll nor are they differentiated into leaves, stems, or true roots. They are widely distributed, especially in the soil.

The role of molds in nature is of very great importance to man. Pathogenic molds are the causes of diseases in plants and animals. Non-pathogenic species include those involved in the breakdown of organic matter in the soil; those concerned in the deterioration and destruction of timber, textiles, foods, and other products; and those with distinct industrial importance, as the molds concerned with the ripening of cheese and the production of commercially valuable organic acids, enzyme preparations, sauces, and related products.

True molds may be divided into four main classes: the *Phycomycetes*, which usually possess nonseptate mycelia; the *Basidiomycetes*, which possess septate mycelia and produce sexual spores exogenously on stalks; the *Ascomycetes*, which possess septate mycelia and produce sexual spores endogenously in sacs, and the *Fungi Imperfecti*, which possess septate mycelia but often produce no sexual spores[1] so far as is now known. (Refer to Fig 72 for subdivisions of these classes.)

General Description.—The individual mold, structurally, may be considered to be made up principally of mycelium and spores. The mycelium is a collection or aggregate of hyphae (singular, hypha), which are thread-like filaments of protoplasm. Hyphae are of two main types: fertile hyphae, which are concerned with the production of reproductive cells or fruit bodies, the spores, and vegetative hyphae, the function of which is to secure nutrient substances from the substrates.

Hyphae may be septate or nonseptate. Septate hyphae are hyphae containing crosswalls or septa, which divide the mold into cells. Nonseptate hyphae contain no crosswalls, but are multinucleate. Molds of the latter type are coenocytic. Cells of septate molds may contain only one nucleus, as in the case of the *Ascomycetes*, or two nuclei, as in the *Basidiomycetes*.

[1] Henrici, A. T., "Molds, Yeasts, and Actinomycetes," John Wiley & Sons, Inc., New York, 1930.

Classes   Subclasses   Orders   Families   Genera

Thallophyta
- Algae — Lichens
- Fungi
  - Myxomycetes (slime molds)
  - Schizomycetes (bacteria)
  - Eumycetes (true molds)

Phycomycetes
- Zygomycetes
  - Mucorales
    - Mucoraceae — Mucor, Rhizopus, Zygorhynchus, etc.
    - Thamnidaceae — Thamnidium, etc.
    - etc.
  - Entomophthorales
  - etc.
- Oomycetes
- Archimycetes

Ascomycetes
- Plectomycetes
  - Plectascales
    - Eurotiales
    - Exoasci — Byssochlamys, Endomyces, Monascus, Saccharomyces, etc.
- Discomycetes
- Pyrenomycetes

Basidiomycetes
- Hemibasidiomycetes
- Protobasidiomycetes
- Autobasidiomycetes

Fungi imperfecti
- Hyphomycetales
  - Mucedinaceae — Aspergillus, Botrytis, Monilia, Oospora, Penicillium, Scopulariopsis, etc.
  - Dematiaceae — Alternaria, Cladosporium, Helminthosporium, Hormodendrum, Stemphylium, etc.
  - etc.
- Melanconiales
- Sphaeropsidales

Fig. 72.—A classification of the fungi. (Courtesy of A. T. Henrici, "Molds, Yeasts, and Actinomycetes," John Wiley & Sons, Inc., New York, 1930; G. Smith, "An Introduction to Industrial Mycology," Edward Arnold & Co., Ltd., London, 1938; H. M. Fitzpatrick, "The Lower Fungi—Phycomycetes," McGraw-Hill Book Company, Inc., New York, 1930;

Molds increase in mass or grow by the extension of the tip cells (apical growth) or in septate types by apical growth and by a division of the cells in any part of the hypha (intercalary growth)

The young cells of a mold are usually filled with dense cytoplasm, but old cells contain many vacuoles and reserve food materials, such as fat globules and glycogen   The cell wall is believed to be composed of chitin

Spores may be asexual or sexual in nature.   Asexual spores may be formed within a closed vessel or spore case known as a "sporangium" (*angium*, "case"), which is supported by a sporangiophore (*phore*, "bearer") in the case of the genera *Mucor* and *Rhizopus*, members of the class *Phycomycetes*, or they may arise from special hyphae, known as "conidiophores"   In the latter case, the spores are known as "conidia."

Asexual spores are borne in various other ways.   The reader interested in this aspect of mycology is referred to some of the texts listed at the end of this chapter.

The chlamydospore is a spore with thickened walls and generally is derived from a vegetative cell

Ascospores are sexual spores and are produced characteristically in a specialized sac or ascus by the class *Ascomycetes*

Zygospores (*zygo*, "yoke") are spores with thick walls formed as the result of the conjugation of two terminal hyphae arising from different colonies, these colonies representing plus and minus strains of the species. Zygospores are thus sexually produced spores.

Distinction is made between molds and a vast number of fungi that are the causative agents of plant diseases and thus live an essentially parasitic life   From the standpoint of industrial microbiology, the term "mold" is generally given to aerobic saprophytes that grow on organic matter or solutions with the formation of expansive masses of mycelium, which may be thin and superficial in character or which may occur as felted masses of tough and/or semigelatinous nature   The mycelia can penetrate the substrate for some distance, especially when growing on cellular tissues or amorphous masses of material

The number of known species of molds is large, but as their classification is often difficult because of great differences in appearance on different substrates it is unwise to attempt exact figures

Molds are especially characterized by the ability to elaborate a great variety of enzymes, and this physiological qualification undoubtedly accounts for their ability to thrive on so many materials and in the presence of very small amounts of organic matter.   Enzyme production by certain species will be considered more fully in a later section of this chapter

The Growth Requirements of Molds.—Certain elements are essential for the growth of molds, such as nitrogen, carbon, hydrogen, oxygen, sulphur, potassium, phosphorus, magnesium, and other elements. Some molds require special organic substances, such as thiamin, for growth

Nitrogen Sources.—Molds, in general, may utilize a large number of nitrogen-containing compounds. These differ in relative value, some stimulating growth by yielding nutritive substances, and some being especially favored on account of their effect in producing large yields of desired end products. The type of nitrogen compound selected is thus of much importance to the fermentologist, who is not only interested in the yield but also in the recovery of the end product in as pure a condition and as free from color as possible.

In general, ammonium salts, nitrates, proteins, peptones, amino acids, and urea are satisfactory sources of nitrogen.

Carbon Sources.—Molds obtain their energy essentially from carbon-containing compounds. In the complete breakdown of a carbohydrate to carbon dioxide and water, as in aerobic respiration, a relatively large amount of energy is liberated. In the anaerobic or partial anaerobic breakdown of carbohydrates, intermediate products are formed which possess less energy value than the original carbohydrate. The energy thus liberated is less than that evolved during aerobic respiration. This type of energy reaction is the one commonly associated with fermentation

Many carbon-containing compounds have been examined as sources of carbon by molds For example, *Aspergillus oryzae*[1] utilized 51 compounds, principally alcohols and acids, for growth and respiration Olive, linseed, and walnut oils; triolein; pentosans; amylopectin; cellulose; some higher paraffins;[2] starches; sugars; and other compounds have been utilized by different molds, in addition to alcohols and acids.

Other Elements.—Iron, zinc, copper, manganese, molybdenum, and gallium appear to be important elements for the growth of certain molds, in particular, *Aspergillus niger*.[3] These elements constitute some of the "trace elements."

Growth Media.—Most molds grow well in a medium containing an appropriate starch or sugar, a usable source of nitrogen, and salts supplying essential mineral elements. An acid reaction is desirable.

Media may be classified as synthetic and natural media. Natural media are obviously the tissues or juices of plants or animals in their native state. It is highly desirable, however, especially for identification

[1] TAMIYA, H., *Acta Phytochim. Japan*, 6: 1 (1932).
[2] BIRKINSHAW, J. H., *Biol. Rev, Cambridge Phil. Soc.*, 12: 357 (1937)
[3] STEINBERG, R. A., *Bull Torrey Botan. Club*, 61: 241 (1934); 62: 81 (1935).
Jour. Agr Research, 51: 413 (1935); 55: 891 (1937). etc ; BIRKINSHAW, *loc cit*.

purposes, to cultivate molds in media that can be duplicated exactly at any time or in different parts of the world. Such synthetic media are prepared with pure sugars and chemically pure inorganic or organic compounds. Steinberg[1] has recently reviewed the subject of the growth of fungi in synthetic nutrient solutions. This excellent paper contains many references to the nutrition of fungi

Raulin's Medium[2]—One of the oldest synthetic media is that of Raulin, which had the following unduly complex composition.

	Grams
Water	1,500
Sucrose	70 0
Ammonium nitrate	4 0
Tartaric acid	4 0
Ammonium phosphate	0 6
Potassium carbonate	0 6
Magnesium carbonate	0 4
Ammonium sulphate	0 25
Zinc sulphate	0 07
Ferrous sulphate	0 07
Potassium silicate	0 07

The foregoing medium has a highly acid reaction (the pH is approximately 2 9)[2]

Czapek's Medium[3]—For growth and isolation of molds Czapek's medium is widely used. This has the following composition, as modified by Dox and Thom.

	Grams
Sucrose	30 0
Sodium nitrate ($NaNO_3$)	2 0
Potassium phosphate (K_2HPO_4)	1 0
Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	0 5
Potassium chloride (KCl)	0 5
Ferrous sulphate ($FeSO_4 \cdot 7H_2O$)	0 01 or trace
Agar	15 (12 to 20)
Water	1 000 cc

The final reaction of the preceding medium is neutral or slightly[2] acid. By using potassium dihydrogen phosphate (KH_2PO_4) instead of potassium monohydrogen phosphate (K_2HPO_4) a definitely acid reaction is obtained. This is preferred by some workers

[1] STEINBERG, R A , *Botan Rev*, **5**: 327 (1939)
[2] SMITH, G , "An Introduction to Industrial Mycology," Edward Arnold & Co , London, 1938
[3] THOM, C , and M B CHURCH, "The Aspergilli," The Williams & Wilkins Company, Baltimore, 1926

Glucose or other sugars, in varying quantities, may be substituted for sucrose in the foregoing formula, thus obtaining a medium with wider applications. Mucors do not grow well on Czapek's medium containing sucrose, for they do not readily utilize this sugar.

In order to avoid a browning of the medium or the production of turbidity, the phosphates should be dissolved separately in a small portion of the water, sterilized, and added to the main portion of the sterilized medium.

Malt Medium.—A medium, liquid or solid, prepared from malt extract is useful where the cultivation of molds and yeasts is concerned. Such a medium has been recommended for the determination of molds and yeasts in butter.

One malt-extract agar is prepared by dissolving 30 g. of dehydrated malt extract (Difco) and 15 g. of agar (Bacto) in 1,000 cc. of distilled water and autoclaving at 15 lb. pressure for 20 min. The final reaction is 5.5.[1]

Sabouraud's Medium.—Parasitic molds grow well in a medium that is a modification of the original formula of Sabouraud.[2] Sabouraud's dextrose agar is prepared by dissolving 10 g. of peptone, 40 g. of dextrose, and 15 g. of agar (Bacto) in 1,000 cc. of distilled water and autoclaving the medium at 15 lb. pressure for 20 min. The final reaction is 5 6[1]

Sabouraud's maltose agar is prepared in the same manner except that maltose is used instead of dextrose. Maltose is more satisfactory for the cultivation of certain pathogenic molds than dextrose, for example, of *Microsporon audouini* and of *M lanosum.*

Liquid media are prepared by omitting the agar. Such media are very useful in certain types of work with molds.

Natural Media.—Prune, potato, carrot, bean, wort, and other juices or extracts with or without agar are sometimes useful in the cultivation of molds.

Nutrient agar or gelatin, such as is used for the growth of bacteria, may be enriched with carbohydrates and adjusted to an acid reaction.

For further data concerning media, the reader is referred to standard texts and publications on mycology and to the reference below.[3]

Methods of Isolating Molds.—There are several methods for isolating molds in pure culture. Some of these are similar to methods used in isolating bacteria or yeasts.

[1] "Difco Manual," Difco Laboratories, Inc , Detroit, 1948.
[2] HENRICI, op. cit.
[3] LEVINE, M , and H W SCHOENLI . ʼ   Media for the
Cultivation of Micro-organisms," The ʼ   ore, 1930

By Agar Dilution.—A series of bacterial culture tubes, perhaps four to six, each containing about 10 cc of a suitable agar medium, is heated in a water bath to melt the agar. The contents of the tubes are cooled to 44 to 42°C. and maintained at that temperature in a water bath until the tubes are used. At this temperature the agar will not solidify, nor will it cause injury to the mold A small amount of the mold-containing material is added to the first of the series of tubes It is mixed thoroughly with the agar by agitation and a small loopful of this seeded agar is transferred to the second tube of the series. The first tube of agar is then poured aseptically into a petri dish The contents of the second tube are shaken carefully, one loopful being transferred to the third tube and the rest poured into a petri dish This process continues until 4 to 6 or more plates have been poured The culture is diluted by this method, and at least one of the plates should contain the desired mold in such dilution that a pure culture may be secured Agar slopes (slants) may be inoculated from this colony.

An alternate method is similar to the above in that tubes of agar are melted and cooled to 44 to 42°C The culture is added to the first tube and after the tube is shaken carefully, the contents are plated. The second tube of agar is then added to the first tube, which contains a few mold spores in the agar still adhering to the sides and bottom of the tube. The latter tube is shaken and the contents poured into a petri dish. A third, fourth, fifth, and perhaps other tubes of agar are added successively to the first tube in the same fashion, and the agar is poured

By Picking Spores from a Single Spore Head.—In this method, the mycologist selects a colony of the mold that he believes is a pure culture and using a hand lens or the low power of the microscope picks mold spores from a single spore head with a sterile needle and transfers them to a tube containing a medium favorable for growth.

If the plate from which the mold colony is selected contains other types of molds, there is a possibility of obtaining a mixed culture

By the Micromanipulator.—This method, though practicable, requires a certain amount of experience Excellent results may be obtained by the use of this method

By the Germination of a Single Spore.—A dilution of spores is made in sterile water or saline until a drop contains just one spore. (Ascertained by an examination of droplets on a slide on the stage of a microscope) Droplets are then placed in isolated portions on the surface of agar, their position being marked in order to locate the correct culture in case the plate contains a contaminant

By a Modification of the Keitt Single-spore Method.—In Ezekial's[1]

[1] Ezekial, W N , *Phytopathology,* **20:** 583 (1930)

modification of the Keitt single-spore method,[1] a nutrient agar, selected for growing the desired mold, is melted, poured into a petri dish to form a thin layer and permitted to solidify. Using a needle (with a spatulate tip) infected with the spore-containing material, which has been diluted properly, four to five parallel streaks are made on the surface of the agar. The dish is then inverted, incubated for 16 to 24 hr., and examined through the bottom with the aid of the 16-mm. objective of a microscope in order to locate a sporeling. When a sporeling has been detected, its position is marked with ink on the plate and it is examined more thoroughly with a high magnification of the microscope. Using a needle with a cylindric tip, a disk of agar containing the sporeling is cut out, placed on a thin portion of the agar on an agar slope and again examined microscopically to make sure that only one sporeling is present.

By the Hansen Method [2]—In another method a dilute suspension of spores is prepared in agar, the agar then being sucked into capillary tubes, the diameters of which are not much greater than the diameters of the spores. The capillary tubes are examined under the microscope. When an isolated spore is found the tube is broken in such a manner that the segment contains a single spore. The glass is treated with alcohol and then placed into fresh medium. Growth emerges from the tube, and a colony develops. This method operates successfully with large, colored spores but not well with small spores.

Single-spore isolations may be made also by the Hansen method or by the Lindner method (refer to Chap. II).

The Identification of a Mold.—The basis for the identification of a mold is an accurate and complete description of the organism. Information obtained by the use of the naked eyes, the hand lens, and the microscope, in the manner outlined in the following paragraphs, is usually sufficient.

It is desirable to cultivate the mold on different types of solid media in order to ascertain the medium best adapted to its growth. Ordinarily Czapek's medium is selected to study the characteristics of a mold that may be used for industrial purposes. This medium has been used in studying the aspergilli and the penicillia by Thom, Church, Raper, and others; in studying the *Actinomycetes* by Waksman; and in studying other molds. A large number of molds grow satisfactorily on this medium. For the sake of comparative purposes it is a good plan to use this medium, then, if the mold grows well on it.

Individual colonies on Czapek's agar or other medium may be studied with the naked eyes, the hand lens, and the low magnification of the

[1] KEITT, G. W., *Phytopathology*, **5**: 266 (1915).

[2] HANSEN, H. N., *Science*, **64**: 384 (1926).

microscope. The following information may be obtained from such observations: the rate of growth; the appearance or growth habit; the nature, arrangement, size, and color of the fruiting bodies and hyphae; the elevation and density of different parts of the colony; the presence or absence of perithecia; variation in the shape and sizes of the mold heads; and other data

The plate may be turned over and the color of the underside of the colony observed, also any coloration produced in the medium

The information gained from the foregoing study may be sufficient to identify the mold insofar as the class or order is concerned, but further study with the aid of the oil-immersion lens of the microscope is usually necessary in order to obtain enough information to identify the mold as to genus and species

Slide cell cultures are very helpful in studies involving the minute structure of molds. Such cultures may be examined either stained or unstained. The following observations are made on the spores: the shape, size (averages and extremes), color, markings, and arrangements. Fertile hyphae are examined in branching, septation, width, color, markings, and the nature of the walls, whether smooth, pitted, or warted. Crystals of chemical compounds or juices elaborated by the mold should be observed

On the basis of the descriptions thus obtained, an attempt may be made to identify the mold, using a text which describes the genera

Fig 73—Diagram of *Aspergillus* (Adapted from Thom and Church. "The Aspergilli," The Williams & Wilkins Company, Baltimore, 1926)

Labels: Conidia; Primary sterigmata; Secondary sterigmata; Vesicle; Stalk or conidiophore; Vegetative cell; Foot cell

Important Genera of Molds.—From an industrial standpoint, species of the genera *Aspergillus*, *Penicillium*, *Rhizopus*, and *Mucor* are by far the most important at the present time. Table 119 summarizes some of the products manufactured on a large scale by the action of molds. It will be noticed that *Aspergillus niger* is particularly important, strains of this organism being used in three important industrial fermentations—the citric, gallic, and gluconic acid fermentations. From amongst the penicillia are species which are important in the ripening of cheeses and which may be used to produce gluconic and citric acids. Species of the genus *Rhizopus* and the genus *Mucor* are of importance in the saccharification of starchy materials. Recent investigations (see Chap XXVII) have

indicated that certain species of *Rhizopus* may soon become valuable in the industrial production of fumaric acid.

TABLE 119.—SOME MOLDS AND SOME MOLD METABOLIC PRODUCTS*

Molds

Aspergillus awamori
A. clavatus
A. elegans
A. flavus*
A. fumaricus
A. fumigatus*
A. giganteus
A. gymnosardae
A. glaucus
A. itaconicus
A. nidulans
A. niger*
A. oryzae*
A. sydowi
A. tamarii
Byssochlamys fulva
Cladosporium sp
Fusarium sp
Helminthosporium penisculatum
Mucor puriformis
M. rouxii*
Paecilomyces divaricatum
Penicillium aurantio-brunneum
P. camemberti*
P. chrysogenum
P. citrinum
P. griseo-fulvum Dierckx
P. javanicum van Beijma
P. purpurogenum
    var. rubrisclerotium
P. roqueforti*
Rhizopus arrhizus
R. chinensis
R. delemar*
R. elegans
R. japonicus*
R. nigricans
R. oryzae
R. pseudochinensis
R. salebrosus
R. shanghaiensis
R. stolonifer
R. tritici
Ustulina vulgaris

Products

Citric acid†
Gallic acid†
Gluconic acid†
Kojic acid
Enzyme preparations†
Mannitol
Ethyl alcohol
Camembert cheese†
Fats
Roquefort cheese†
Fumaric acid
Lactic acid
Saccharified starch†

* Mold species of present industrial importance.
† Products produced on an industrial basis by mold action.

Products Formed by Molds.—Table 120 lists some of the products formed by molds. Many of these are of a complex nature. The antibiotics produced by molds are listed in Chap. XXXVI.

Characteristics of the Genus Aspergillus.—The mycelium consists of septate branching hyphae, which may be brightly colored or colorless

TABLE 120.—SOME PRODUCTS FORMED BY MOLDS

Acids	Alcohols	Enzymes	Pigments	Polysaccharides	Sterols	Miscellaneous
Acetic	s-Erythritol	Amalase	Aspergillin	Caprochaose	Cholesterol	Acetaldehyde
Aconitic	Ethyl	Amylase (α & β)	Aurantin	Galactocarolose	Ergosterol	Alboleersin
Allantoic	Glycerol	Carboxypoly	Aurofusarin	Glycogen	Fungsterol	Dimethyl
Byssochlamic	Mannitol	peptidase	Aurofusarin	Gums	Phytosterol	selenide
Carbic		Catalase	Boletol	Luteic acid		Ergot
Carbolic		Cellulase	β-Carotene	Mannocarolose		Ergosteryl
Cardic		Cytase	Carvomsin	Mycodextran		palmitate
Carolinic		Dextranase	Carvolin	Polygalactose		Ethyl acetate
Citric		Dipeptidase	Catenarin	Poly mannose		Griseofulvin
3'5-Dihydroxy-		Emulsin	Chrysoanin	Rugulose		Hydroxylamine
phthalic		Fropsin	Citrinin	Sclerotiose		Lipoin
Dimethyl pyruvic		Gentianase	Citromycetin	Starch (mold)		Pahtamin
L-Ethylene oxide		α-Glucosidase	Cudbearin	Trehalose		Phenylethyl
α,β dicarboxylic		β-Glucuronidase	Cynodontin	Varianose		amine
Formic		Histozyme	Emodic acid			Terrein
Fulvic		Inulase	Emodin mono-			Vitamins
Fumaric		Invertase	methyl ether			Etc
Fusarinic		Lactase	Erythroglaucin			Arsenic com-
Gallic		Lecithinase	Flavoglaucin			pounds
Gentisic		Lipase	Fulvic acid			Methyldi-
Glauconic		Maltase	Funiculosan			ethylamine
d-Glucaric		Melezitase	3-Hydroxy			Dimethyl-
Gluconic		Nuclease	emodin			alkylamine
Glycollic		Protease	Hydroxy molch-			Trimethyl-
Glyoxylic		Raffinase	mitchorporin			arsine, etc
2 Hydroxy methyl		Rennet	Luteoharsin			Chlorine-con-
furane-3-car		Sulphatase	Monascoflavin			taining
boxylic		Tannase	Monascorubin			compounds
Isovaleric		Trehalase	Ochracin			Calatomycin
Itaconic		Urease	O sporin			Frdin
Itatartaric		Zymase	Penicinor acid			Grodin
Kojic			Penicillopsin			Griseofulvin
γ-Ketopentadecenoic			Phoenicin			Sclerotiorine
Lactic			Physcion			
Luteic			Ravenelin			
Malic			Rubrofusarin			
Malonic			Rubroglaucin			
d-Mannonic			Tritisporin			
l-γ-Methyltetronic						
Methyl salicylic						
Mintoluteic						
Oxalic						
Penicillic						
Puberulic						
Pyruvic						
Speculosporic						
Stipitatic						
Succinic						
Terrestric						

and which may produce crusts or sclerotia.[1] The mycelium is usually partially submerged in the substrate and partially aerial.

The foot cell is a specialized, enlarged, thick-walled cell that gives rise to a fertile hyphae, *i.e.*, the stalk or conidiophore. The foot cell is usually, but not always, submerged in the substrate.

The stalk, or conidiophore, arises approximately perpendicularly to the long axis of the foot cell. Its walls may be smooth, pitted, or rough. It may be septate or unseptate. At the apex, the stalk usually enlarges to form a vesicle.

The vesicle, which supports the sterigmata, is globose, hemispherical, elliptical, clavate, calyptrate, or of other shape. A portion or all of its surface is covered with sterigmata.

Sterigmata (singular, "sterigma") produce conidia or clusters of other sterigmata. When there are two series of sterigmata present, the first series, the one adjacent to the vesicle, is designated as the "primary sterigmata." These give rise to and support the second series of sterigmata, which are called "secondary sterigmata." In the latter case, conidia are produced by the secondary sterigmata.

The conidium (spore) is produced by an elongation and cell division of the sterigma. A crosswall appears and the newly formed cell matures. Other conidia are produced by the same sterigma in a similar manner with the result that an unbranched chain of conidia appears, of which the outermost are the eldest. These conidia vary among different species in respect to color, size, and shape.

Spore heads vary in respect to arrangement, color, size, and shape. Heads may be globose, like *A. niger*; hemispherical, like *A. terricola* var. *americana*; elliptical; clavate, like *A. clavatus* (the head in this case is clavate or elliptical); columnar, like *A. flavipes*; or of some other characteristic shape.

Perithecia are produced by only a few species. The perithecium[2] is a thin-walled receptacle, or fruit body, commonly globose or flask-shaped, closed at maturity, which produces ascospores. The ascospores are liberated when the thin walls of the perithecium break.

Sclerotia, which are hard masses formed from the mycelium, usually possess somewhat characteristic markings and colorations. They are produced by some species of *Aspergillus*.

For a further discussion of this subject the reader is referred to the "Manual of the Aspergilli," by Thom and Raper (1945).

Some Important Aspergilli.—Members of the *A. flavus-oryzae* group, especially strains of the *A. oryzae* series, have large industrial importance,

[1] Thom and Church, *loc. cit.*

[2] Smith, *op. cit.*

particularly in the Orient. In Japan, *A. oryzae* is used to saccharify rice starch in the manufacture of sake and other alcoholic liquors, in the manufacture of *shoyu* (soy sauce); in the manufacture of *miso*, a soybean product used as a breakfast food, and in the preparation of *mizaume*, a sugar sirup made from rice. *A. oryzae* is used also in the preparation of enzyme mixtures, which appear on the market under such trade names as Takadiastase, Polyzyme, Digestin, Oryzyme, and Kashwagidiastase. Kojic acid is produced by the same mold.

Strains of *A. niger* are used in three industrial fermentations (refer to Chaps. XXV, XXVI, and XXVIII). This mold may cause serious damage in the textile industry (see Chap. XXXVII).

A. tamarii is used in the Orient in the production of *tamari* sauce from soybeans or from soybeans mixed with rice.

Fig. 74.—A division of the genus *Penicillium*.

Divisions of the Penicillia.—The penicillia are divided into four main groups by Thom: the Monoverticillata, the Biverticillata-symmetrica, the Polyverticillata-asymmetrica, and the Asymmetrica.

[Note C.] The Penicillia. The Wild and Biverticillata-symmetrica Penicillia.

Polyverticillata-symmetrica, **and the** *Asymmetrica.* These groups are still further divided in some cases. The basis for the principal divisions is the type of branching in the penicillus or spore head. Colony characteristics furnish a basis for further subdivision.

Figure 74 shows a division of the penicillia proper into main groups Some important species are listed.

The *Monoverticillata* contain one cluster, whorl, or verticil of sterigmata (the conidia-producing organs of the mold) supported by the conidio-

phore. In this group belong the *Citromyces* of Wehmer, molds that have the ability to produce citric acid; and *P. Charlesii* and *P. spinulosum,* molds studied for their biochemical characteristics by Raistrick and his associates.

In the *Biverticillata-symmetrica,* a verticil or whorl of sterigmata is supported by short branches or metulae (singular, metula), which are arranged symmetrically about the axis of the conidiophore in the form of a verticil or whorl. *P. luteumpurpurogenum,* a mold that produces gluconic acid, and *P. pinophilum,* a mold that stains wood,[1] are members of this group

There are three or more stages of branching in the *Polyverticillata-symmetrica* group, which contains only a few unimportant molds.

FIG 75.—Diagram of the penicillus of a *Penicillium* (Adapted from Thom, "The Penicillia," The Williams & Wilkins Company, Baltimore, 1930)

Labels in figure: Conidia, Sterigmata, Metulae, Ramulus, Ramus, Conidiophore

In the *Asymmetrica,* the spore head, or penicillus, is branched asymmetrically about the axis of the conidiophore. This group is the largest of the four and includes species of economic importance: molds producing characteristic changes in cheeses; molds causing destruction of fruits; and molds with the ability to produce gluconic acid or other compounds from nutrient glucose solutions.

Two species of *Penicillium,* *P. italicum* and *P. digitatum,* cause much damage to citrus fruits. Invasion of the fruit occurs through wounds. Careful handling and packing, the use of special protective treatments[2] to prevent infection of the fruit, and the use of wrapping material treated with diphenyl[3] do much to prevent losses due to these molds

[1] HENRICI, *loc. cit.*
[2] PRESCOTT, S. C., and B. E. PROCTOR, "Food Technology," McGraw-Hill Book Company, Inc, New York, 1937
[3] *Ind. Eng Chem. (News Ed)* 17: 210 (1939)

P. italicum produces a soft rot of citrus fruits. The colonies on the fruit are blue-green in color owing to the spores.

P. digitatum (P. ~~....~~ ~~....~~ .... olive-green in color.

up.[1] Conidia of th .....

Soft rot of store~~c~~ ~~..~~pcs and pears is caused by *P. expansum*. Coremia formation is characteristic of the mold. The coremia, *i.e.*, bundles of conidiophores, are green to gray-green in appearance.

The Genus Rhizopus.—Species of *Rhizopus* are of industrial value to man *Rhizopus oryzae* and other species have the ability to produce
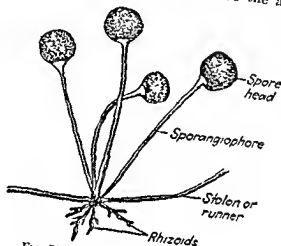
Fig 76 —Diagram of *Rhizopus nigricans*

d-lactic acid from nutrient sugar media (see Chap XXXI) while *R. japonicus, R. delemar*, and other species have been used in the Amylo process for converting starches to sugars (see page 90)

On the other hand, certain species of *Rhizopus* are the cause of the destruction of plant products *R. nigricans* is the cause of soft rot in sweet potatoes, especially during storage The same mold also causes rot in fruits, for example, the soft watery rot of strawberries, which is known also as "leak" *R necans* produces a bulb rot in the lily, *R nodosus* a boll rot of cotton.[2]

General Characteristics of the Genus—Reference to the accompanying figure will aid in understanding the general description of the genus *Rhizopus*. Erect aerial sporangiophores arise from th~~.~~ .... where the stolons or runn~~...~~ .... upper ends of the by means of rhizoids. ..... .

[1] SMITH, *op cit*
[2] HEALD, F D, "Introduction to Plant Pathology," 2d ed , McGraw-Hill Book Company, Inc , New York, 1943.

fruiting hyphae (sporangiophores) in clusters or whorls. Groups of sporangiophores are connected by hyphae of a vegetative nature, the stolons, which may be regarded as distributing hyphae. The rhizoids are vegetative hyphae, which function as anchors.

TABLE 121.—SOME ENZYMES FORMED BY SOME INDUSTRIALLY IMPORTANT MOLDS

Aspergillus niger	*Aspergillus oryzae*	*Penicillium camemberti*
Amylase (diastase)	Amidase	Amidase
Cellobiase	Amylase	Amylase
Emulsin	Catalase	Emulsin
Gentianase	Cytase	Erepsin
Gentiobiase	Dextrinase	Inulase
Inulase	Emulsin	Invertase
Invertase	α-Glucosidase	Lactase
Lipase	β-Glucosidase	Lipase
Maltase	Histozyme	Maltase
Melezitase	Inulase	Nuclease
Nuclease	Invertase	Protease
Protease	Lactase	Raffinase
Raffinase	Lecithinase	
Rennet	Lipase	
Tannase	Maltase	
Trehalase	Protease	
Zymase	Rennet	
	Sulphatase	
	Tannase	

Thick-walled spores (chlamydospores) are formed by some species, for example, *R. oryzae*.

Mold Enzymes.—Various kinds of enzymes are produced by different species of molds. A list of some of the enzymes produced by strains of the *Aspergillus niger* and *A. oryzae* groups and *Penicillium camemberti* is given in Table 121.

With certain exceptions, molds have not been thoroughly studied in respect to the kinds of enzymes elaborated and the conditions under which they are produced. This field of research offers many possibilities.

The interested reader will find considerable information dealing with the enzymes of molds in the text prepared by Waksman and Davison.[1]

Apparatus for Cultivating Molds.—From time to time special equipment has been devised for cultivating molds or for carrying out mold fermentations on a large scale in the laboratory. Birkinshaw and his associates have described an incubator for the large-scale growth of

[1] WAKSMAN, S. A., and W. C. DAVISON, "Enzymes," The Williams & Wilkins Company, Baltimore, 1926.

molds.[1] An annation
has been d......

...... A sterilizer-incuba-
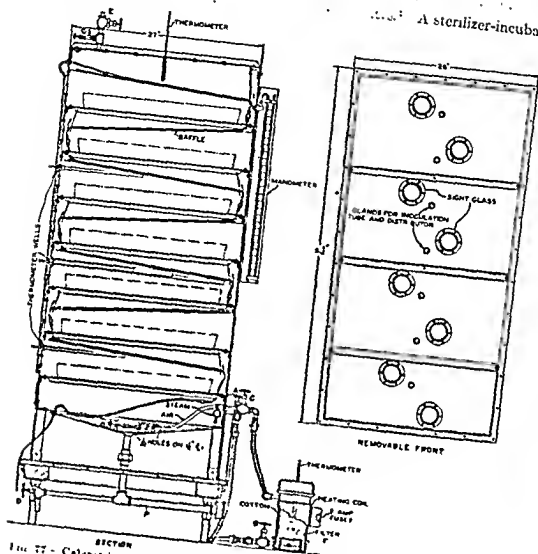
Fig 77 - Calmet incubator [From Bird Lockwood May and Hermel Ind Eng Chem, 27: 318 (1935)]

tor, somewhat similar to the ones mentioned above, was described by Ward and his collaborators[4] (see Fig 77)

[1] BIRKINSHAW, J B, J H V CHARLES, C H LILLY, and H RAISTRICK, Trans Roy Soc (London), B220: 136 138, 355 367 (1931)
[2] PETERSON, W H, L M PRUESS, H J GORTNER, and H C GREENE, Ind Eng Chem 25 213 (1933)
[3] WARD, G E, L B LOCKWOOD, O E MAY H T HERRICK, Ind Eng Chem, 27: 318 (1935)

In the chapter on the gluconic acid fermentation, other types of equipment used in fermentation are described or referred to.

References on Molds

BESSEY, E A : "A Textbook of Mycology," P. Blakiston's Son & Company, Philadelphia, 1935   (This book contains a chapter entitled, "Guide to the Literature for the Identification of Fungi," which should prove of much value.   An extensive bibliography is presented.)

CHALLENGER, F : Some Fermentative Changes of Technical Interest, *Ind. Chemist*, **61**: 97 (1930)

CLEMENTS, F E., and C. L. SHEAR: "The Genera of Fungi," The H. W. Wilson Company, New York, 1931

DE BARY, A : "Comparative Morphology and Biology of the Fungi, Mycetozoa and Bacteria," Oxford University Press, New York, 1887

DODGE, C W.: "Medical Mycology," The C. V. Mosby Company, St. Louis, 1935

ELLIOTT, J A.: Taxonomic characters of the genera *Alternaria* and *Macrosporium*, *Am Jour. Botany*, **4**: 439 (1917).

FITZPATRICK, H M.: "The Lower Fungi—Phycomycetes," McGraw-Hill Book Company, Inc., New York, 1930

GÄUMANN, E A., and C W DODGE: "Comparative Morphology of Fungi," McGraw-Hill Book Company, Inc., New York, 1928

GILMAN, J. C.: "A Manual of Soil Fungi," The Iowa State College Press, Ames, Iowa, 1945.

GUILLIERMOND, A.: "The Yeasts" (translated and revised by F W. Tanner), John Wiley & Sons, Inc , New York, 1920.

GWYNNE-VAUGHAN, H. C. I.: "Fungi," The Macmillan Company, New York, London, 1922.

——— and B. BARNES: "The Structure and Development of Fungi," 2d ed , The Macmillan Company, New York, 1938.

HARSHBERGER, J W.: "Mycology and Plant Pathology," P. Blakiston's Son & Company, Philadelphia, 1917.

KRIEGER, L C C.: "The Mushroom Handbook," The Macmillan Company, New York, 1936

LAFAR, F : "Handbuch der technischen Mykologie," 2d ed , Gustav Fischer, Jena, 1904-1914.

———: "Technical Mycology," Charles Griffin & Company, Ltd , London, 1898-1910

LEONIAN, L H , and V G. LILLY: Thiamin in the Nutrition of Fungi, Abstracts of Communications, Third International Congress of Microbiology, p 201, New York, Sept. 2-9, 1939.

LINDAU, G : "Kryptogamenflora fur Anfänger," Band II, "Die mikroskopischen Pilze," 2d ed , Verlag Julius Springer, Berlin, 1922.

LUTMAN, B. F : "Microbiology," McGraw-Hill Book Company, Inc., New York and London, 1929

MAY, O E., and G E. WARD: Hydrolysis of the Chitinous Complex of Lower Fungi, *Jour. Am Chem. Soc.*, **56**: 1597 (1934).

NIELSEN, N : The Growth Substance Demand of Different Fungi Abstracts of Communications, Third International Congress of Microbiology, p 201, New York, Sept 2-9, 1939.

Rabenhorst, L.: "Kryptogamenflora von Deutschland, Oesterreich und der Schweiz," Edward Kummer, Leipzig, 1884–1921

Ramsbottom, J.: "Fungi," Ernst Benn, Ltd., London, 1920

———: "The Uses of Fungi," address to Section K (Botany) of the British Association for the Advancement of Science at Blackpool, Sept. 10, 1936, *Annual Report*, p. 189, 1936

Raper, K. B., and D. F. Alexander: Presentation of Molds by the Lyophil Process, *Mycologia*, 37 (No. 4): 499–525 (1945)

Saccardo, P. A.: "Fungi Italici autographice delineati," Paris, 1877–1880

———: "Sylloge fungorum omnium hucusque cognitorum," Pavia, 1882–1931

Salle, A. J.: "Fundamental Principles of Bacteriology," McGraw-Hill Book Company, Inc., New York, 1939

Shaw, F. W.: Fungi and Fungous Diseases, *Stuart Circle Hosp. Bull.*, August, 1932

Skinner, C. E., C. W. Emmons, and H. M. Tsuchiya: "Henrici's Molds, Yeasts, and Actinomycetes," 2d ed., John Wiley & Sons, Inc., New York, 1947.

Smith, G.: "An Introduction to Industrial Mycology," Edward Arnold & Company, London, 1938

Steinberg, R. A.: The So-called "Chemical Stimulation" of *Aspergillus niger* by Iron, Zinc and Other Heavy Metal Poisons, *Bull. Torrey Botan. Club* 61: 211 (1934)

——— The Nutritional Requirements of the Fungus *Aspergillus niger*, *Bull. Torrey Botan. Club*, 62: 81 (1935)

——— Nutrient-solution Purification for Removal of Heavy Metals in Deficiency Investigations with *Aspergillus niger*, *Jour. Agr. Research*, 51: 413 (1935)

——— Role of Molybdenum in the Utilization of Ammonia and Nitrate Nitrogen by *Aspergillus niger*, *Jour. Agr. Research*, 55: 891 (1937)

——— Growth of Fungi in Synthetic Nutrient Solutions, *Bot. Rev.*, 5: 327 (1939)

Stevens, F. L.: "Plant Disease Fungi," The Macmillan Company, New York, 1925

Thom, C.: "The Penicillia," The Williams & Wilkins Company, Baltimore, 1930

——— and M. B. Church: "The Aspergilli," The Williams & Wilkins Company, Baltimore, 1926

——— and M. Phillips: Chemistry—Legumin-like complexes in fungi, *Jour. Wash. Acad. Sci.*, 22: 237 (1932)

——— and K. B. Raper: "A Manual of the Aspergilli," The Williams & Wilkins Company, Baltimore, 1945

Waksman, S. A., and W. C. Davison: "Enzymes," The Williams & Wilkins Company, Baltimore, 1926

Wolf, F. A., and F. T. Wolf: "The Fungi," Vols. I and II, John Wiley & Sons, Inc., New York, 1947

Wollenweber, H. W., and O. A. Reinking: "Die Fusarien," Paul Parey, Berlin, 1935

———, C. D. Sherbakoff, O. A. Reinking, H. Johann, and A. A. Bailey: Fundamentals for taxonomic studies of Fusarium, *Jour. Agr. Research*, 30: 833 (1925)

Zycha, H.: "Kryptogamenflora der Mark Brandenburg," Band VI a, "Pilze II, Mucorineae," Verlagsbuchhandlung Gebrüder Borntraeger, Leipzig, 1935

CHAPTER XXV

THE CITRIC ACID FERMENTATION

The production of citric acid by fermentation on a commercial basis has been a highly important achievement in the field of industrial microbiology. It has made the United States self-sufficient in respect to the citric acid supply and greatly changed the commerce of the world in citric acid and calcium citrate.

Citric acid (COOH·CH₂·C(OH)·COOH·CH₂·COOH) was first isolated from lemon juice and crystallized as a solid by Scheele in 1784. It is found as a natural constituent of citrus fruits, pineapples, pears, peaches, figs, and other fruits and tissues. The citric acid extracted from these products is known as natural citric acid in contrast to fermentation citric acid. Cull lemons, limes, and pineapples are the principal sources of natural citric acid, which is produced chiefly in Italy, especially Sicily, and also in California, Hawaii, and the West Indies

In 1922, Italy produced approximately 90 per cent of the world's supply of calcium citrate, which is used in citric acid manufacture. Much of this calcium citrate was exported to the United States, England, and France. Since 1927, however, very little calcium citrate or citric acid has been imported into this country. Several factors have been responsible for this change: the production of citric acid by mold fermentation, an increase in the numbers of trees bearing lemons in the United States, importations of concentrated lemon juice, and high import duties.

Wehmer, in 1893, first described citric acid as a product of mold fermentation. Two molds, which he designated as *Citromyces pfefferianus* and *Citromyces glaber* (classified by Dr. Thom as penicillia), produced acid from nutrient sucrose solutions containing calcium carbonate. Later Wehmer reported the formation of citric acid by *Penicillium luteum* and *Mucor piriformis*, but it is interesting to note that he believed that the black aspergilli produced only oxalic acid. This idea was disproved by Thom and Currie. Nevertheless, oxalic acid is generally an accompaniment of the citric acid produced.

In 1917, Currie, of the U S. Department of Agriculture, published the results of a fundamental research concerning the production of citric acid by a strain of *Aspergillus niger*. Doelger and Prescott, in 1934, corroborated the results of Currie and made other valuable contributions concerning the fermentation

The literature of the past two decades contains many references to the citric acid fermentation, but no attempt will be made to review it in detail. Certain selected references giving the principal facts gained by research will be found at the end of this chapter. The interested student is urged to consult some of the papers cited.

Significant Factors in Fermentation.—The organism, the correct interrelation of the various constituents of the medium—sugar and inorganic salts—the pH, the ratio of surface area to volume of solution fermented, the oxygen supply, and the temperature have much to do with the nature and magnitude of the yield of end products recovered from a fermentation medium. By adjusting the salts and pH carefully, it is possible to produce citric acid with a negligible or small amount of oxalic acid.

Organisms.—Since the historic researches of Wehmer, it has been shown that a large number of fungi have the ability to produce citric acid
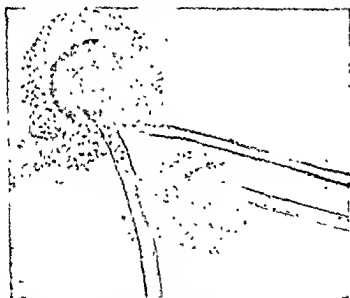
Fig. 78 — Typical spore-bearing heads of *Aspergillus niger* Club-shaped head bears two rows of columnar cells with spores forming at the ends of the second row of cells. [From B P Dodger and S C Prescott, Ind Eng Chem , 26 1142 (1911)]

Some of the fungi produce small yields; some produce undesirable substances, some, on account of their unstable cultural characteristics, would be unsatisfactory for use on a commercial basis Thus the choice of a strain is of great importance

Aspergillus niger, *A clavatus*, **Penicillium luteum**, *P. citrinum*, *Paecilomyces divaricatum*, *Mucor piriformis*, *Ustulina vulgaris*, and another

species of *Mucor* have been used to produce citric acid in the laboratory or on a commercial scale.

Strains of the *Aspergillus niger* group of molds have usually given most successful results, both in the laboratory and on an industrial basis Many of these molds produce high yields, possess fairly uniform biochemical characteristics, are easily cultivated, and produce a negligible quantity of undesirable end products.

Sugar.—Many organic substances, among them 2-, 3-, 4-, 5-, 6-, 7-, and 12-carbon compounds (principally sugars), may be fermented to citric acid. Maximum yields have been secured, usually, from sucrose and fructose. Occasionally glucose, under certain circumstances, has given high yields, comparable with those from sucrose. For industrial fermentations, sucrose and technical glucose are best; maltose and molasses are less desirable.

In general, a high concentration of sugar is required to produce high yields of citric acid. Solutions with concentrations of 14 to 20 per cent may be used Currie advocated the use of 125 to 150 g. of sucrose per liter. Doelger and Prescott obtained highest yields when using a concentration of 140 g. of sucrose per liter in fermentations that were allowed to run for 9 to 12 days. They found that if more than 15 per cent of sugar was used, a greater amount of sugar than normal (less than 3 per cent) remained unconverted to citric acid. Substitution of part of the sucrose by fructose or glucose, such that the concentrations of these sugars represented 1 to 5 per cent (out of the total of 14 per cent) resulted in lower yields of citric acid than were obtained from controls containing sucrose alone. Partial hydrolysis of sucrose during sterilization likewise resulted in lower yields.

Inorganic Salts—In addition to the carbon, hydrogen, and oxygen supplied by the carbohydrate, also nitrogen, potassium, phosphorus, sulphur, and magnesium are indispensable in the fermentation medium, according to Currie and Doelger and Prescott Currie[1] found that the most favorable medium for producing citric acid contained the following.

	Grams per Liter	
Sucrose	125	–150
NH₄NO₃	2	– 2 5
KH₂PO₄	0 75–	1 0
MgSO₄·7H₂O	0 20–	0 25
HCl to pH 3 4–3 5 (5–4 cc N/5 HCl)		

Doelger and Prescott[2] found the following medium to be most satis-

[1] Currie, J N, *Jour. Biol. Chem.,* **31:** 15–37 (1917).

[2] Doelger, W. P., and S C. Prescott, *Ind Eng Chem.,* **26:** 1142 (1934)

factory, for the strain of *Aspergillus niger* used produced high yields of
citric acid with less than 2 per cent of oxalic acid from this medium:

	Grams per Liter
Sucrose	140
NH$_4$NO$_3$	2 23
K$_2$HPO$_4$	1 00
MgSO$_4$.7H$_2$O	0 23

(Salts and sugars are dissolved and made up to 1 liter with distilled
water, adjusted to pH 2.20 to 1 60 with N/1 HCl, and sterilized at 8 to
10 lb steam pressure per square inch for 30 min.)

If more than 2.50 g of ammonium nitrate, 1.50 g. of potassium mono-
hydrogen phosphate and 0.30 g of magnesium sulphate were used, oxalic
acid formation increased and the yield of citric acid was decreased.
Ammonium nitrate in a concentration of more than 2.50 g. per liter caused
the formation of a heavy mat. More than 0.30 g. of magnesium sulphate
per liter favored sporulation. In general, high yields of citric acid were
obtained when the mats were thin and the sporulation light or nearly
absent; these results were secured when a minimum quantity of inorganic
salts were used. Restriction of the nitrogen supply tended to cause
increased yields of citric acid

Wells and Herrick[1] report the following limits for the amounts of salts
generally used in the fermentation. 0 03 to 0.1 per cent of KH$_2$PO$_4$,
0 01 to 0 05 per cent of MgSO$_4$.7H$_2$O, and 0 16 to 0 32 per cent of
NH$_4$NO$_3$

In certain cases, other nitrogen-containing salts have been found
to be superior to ammonium nitrate. Sodium nitrate in a 0 4 per
cent concentration was found to be better than ammonium nitrate or ammo-
nium sulphate by Porges, while Butkewitsch and Gaewskaya used
potassium nitrate in a 0 35 per cent concentration in order to obtain
high yields of citric acid

The use of salts other than those mentioned above does not usually
appear to be necessary, although many apparently conflicting state-
ments concerning the value of the use of certain salts in the medium for
producing high yields of citric acid appear in the literature For example,
some investigators state that iron and zinc accelerate the formation of
citric acid; others are of the opinion that salts of iron and zinc stimulate
growth of the mycelium without increasing the yields Still others
have shown that zinc salts have a definite inhibitory effect on citric acid
production Thus, in a few instances, it seems that iron, and possibly
zinc salts, have favored acid production

[1] WELLS, P A, and H T HERRICK, *Ind Eng Chem*, 30: 255 (1938)

Undoubtedly, the strain of fungus used by a worker has a very important bearing on the salt requirement. That this is true has been shown by several men. Osnizkaya, using one strain of *Aspergillus niger*, obtained a marked increase in the yield of citric acid from sucrose by the addition of 0.3 per cent magnesium nitrate, but, when using a second strain, the addition of magnesium nitrate actually caused a slight diminution in the yield. According to Quilico and Di Capua, the effect of iron on citric acid production depends on the strain of *A. niger* used. In one case the yield of citric acid was increased and in another case decreased by the addition of increasing quantities of iron.

Perlman, Dorrell, and Johnson[1] studied the effects of the addition of metallic ions to two highly purified media on the production of citric acid by the surface culture method, using five strains of *A. niger*. They concluded, on the basis of many experiments, that the optimum concentration of a given metallic ion for the stimulation of citric acid production varied with the strain of *A. niger* used. For example, the addition of 0.1 mg. of iron per liter to one fermentation medium produced optimum results in the case of *A. niger* 62; the addition of between 0.1 and 1.0 mg of iron per liter to a second fermentation medium was optimum for strains 62, 69, 70, and 72; and the addition of 10 mg. of iron per liter appeared to be optimum for strain 59. When other stimulatory ions were used together with iron, the results were in general no better than those obtained with iron alone. However, in the case of strain 62, some combinations of iron and manganese ions produced better yields than either ion alone.

Aluminum, chromium, iron, and manganese ions were the only ones that stimulated acid production with *A. niger* 62. The other ions used, molybdenum, copper, zinc, and calcium, were inhibitory to acid production in the concentrations used. Molybdenum stimulated acid production by strain 72, while iron was somewhat inhibitory.

The presence or absence of minute traces of elements in a medium may have a marked effect on the result obtained. Improved methods of analysis—the use of the spectroscope, and other precision measurements—have aided the microbiologist and chemist greatly in recent years in detecting the presence of unsuspected elements in supposedly pure compounds. An increasing amount of evidence stresses the importance of the presence of mere traces of substances, in quantities of a fraction of a part per million, for example.

pH.—The maintenance of a favorable pH is most important for the successful progress and termination of a fermentation. Currie demonstrated

[1] PERLMAN, D, W. W. DORRELL, and M. J. JOHNSON, *Arch. Biochem.*, **10** (No 3): 131 (1946)

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strated that by controlling the pH and the inorganic salts, the proportions of citric and oxalic acids could be varied considerably. In fact, when using conditions that favored the highest yields of citric acid, the formation of oxalic acid was nearly completely suppressed

The use of a low pH is advantageous in that high yields of citric acid are favored, oxalic acid formation is suppressed, and the danger of contamination is minimized. Sterilization is more readily effected at a low pH. In the laboratory, successful fermentations have been carried out without resorting to heat sterilization of the medium when the initial pH was low (2.20 or below) In general, the best citric-acid-producing molds possess the greatest tolerance to low pH values

Hydrochloric acid was used by Currie to adjust the pH of his medium to 3 1 to 3.5. Doelger and Prescott advocated the use of this acid also, for the element chlorine was found to be of distinct value as a constituent of the medium. The pH range of 1 60 to 2 20 was found to be the most satisfactory by these men; a pH value in the upper part of this range was used when distilled water was employed in the medium rather than Cambridge tap water This indicates that for commercial production the character of the water supply should be carefully observed. From the point of view of yields of citric acid, sulphuric, nitric, and acetic acids were found to be inferior to hydrochloric acid. A quantity of formic acid sufficient to lower the pH to 3 0 prevented the mold spores from germinating

It is not generally considered necessary to add calcium carbonate to neutralize the acids formed during fermentation Its use favors contamination, while its absence favors higher yields and a shorter fermentation period However, calcium carbonate has been used by Wehmer, Chrzaszcz, and Peyrous to increase the yield of citric acid Here again the strain of *Aspergillus niger* used and its tolerance to acid may be of large significance

Ratio of Surface Area to Volume—In the citric acid fermentation, the conversion of sugar to citric acid is brought about by intracellular enzymes and therefore takes place within the living cells that make up the mycelial mat Sugar passes by osmosis into the cells while the acid diffuses out through the cells The rate at which enzyme and diffusion processes proceed will determine the length of the fermentation period Obviously, in a deep vessel containing a large volume, the progress of acid formation will be relatively slow for the surface area of the mat will be small in comparison with the volume By using shallow pans, a large surface area of mycelium is exposed to a relatively shallow layer of medium Conversion of sugar to citric acid proceeds much more rapidly [   ] with a maximum quantity of

The ratio of surface area to volu...

citric acid will be produced during the shortest fermentation period with a minimum of sugar unconverted to citric acid should be used.

The effect of varying the ratio of the volume to the surface area of the medium is illustrated in the following table from Doelger and Prescott's researches. Shallow pans of aluminum (25 by 33 cm.), of a purity of 99.80 per cent or greater, were used during the experiment. Standard medium at a pH of 2.40 was inoculated and maintained at a temperature of 26°C. until the ninth day, when analyses were carried out.

TABLE 122.—THE EFFECT OF VARYING THE RATIO OF VOLUME TO SURFACE AREA ON THE YIELDS OF CITRIC ACID[1]

Volume: surface area ratio, cc. per sq. cm.	Original volume, cc	Sucrose per pan, grams	Final volume, cc.	Citric acid per 100 cc, grams	Total citric acid per pan, grams	Yield of citric acid per sugar, per cent
2 45	2,000	280	1,810	6.35	114 9	41 0
2.20	1,800	252	1,620	7.05	114 2	45 3
2 08	1,700	238	1,490	7.40	110.3	46 3
1.83	1,500	210	1,310	7 75	110 2	48 3
1 22	1,000	140	780	8.80	68.6	49.0

[1] DOELGER, W. P., and S. C. PRESCOTT, *Ind Eng. Chem.*, **26:** 1142 (1934).

Agitation of the medium by a gentle or moderate shaking motion retards the rate of citric acid production, according to Doelger and Prescott. (Compare with the sorbose and dihydroxyacetone fermentations when the shallow-pan method is used.)

Oxygen Supply.—Large amounts of air have an adverse effect on the yield of citric acid, according to Porges, Doelger and Prescott, and Gudlet The flow of small amounts of air over the mat has no injurious effect, however, as Wells and his associates have passed sterile, humidified, carbon-dioxide-free air over mats in 2,000-cc. Erlenmeyer flasks at a rate of 15 cc. per min. and obtained results similar to those secured with controls receiving no special air supply. Too low an air supply, on the other hand, also reacts unfavorably on the yield of citric acid. Regulation of supply of air must therefore be determined experimentally for each installation of apparatus.

Temperature.—The temperature used will depend in part on the organism and the fermentation conditions. Temperatures of 25 to 35°C. are usually employed. Doelger and Prescott advocate 26 to 28°C as the optimum temperature range. They state that the "amount of citric acid produced will be on a rising scale as the temperatures are increased from 8 to 28°C " and that at 30°C. or above "citric acid produc-

tion will decrease and a greater proportion of the titrable acidity will be due to the formation of oxalic acid." The following table shows the effect of the temperature of incubation on titrable acidity and the quantity of citric acid formed. Standard medium was used in the experiment.

In 250-cc. flasks were placed 75-cc. portions, and all were inoculated from the same 10-day-old culture of mold spores (*Aspergillus niger*).

After a 10-day period of incubation, samples from each flask (10 at each temperature) were analyzed.

TABLE 123.—EFFECT OF TEMPERATURE OF INCUBATION ON TITRABLE ACIDITY AND CITRIC ACID PRODUCED[1]

Temp of incubation, °C	Titrable acidity, normality	Citric acid produced		Evaporation of medium, per cent
		Per flask fermentation, grams	Per 100 g sugar, grams	
20–22	1 0024	3 37	32	24
24	1 0535	3 55	34	27
26	1 1187	3 96	38	26
28	1 1564	3 88	37	30
30–33	1 1045	2 87	27	36

[1] DOELGER W P and S C PRESCOTT, *Ind Eng Chem*, **26**: 1142 (1934)

Duration of Fermentation Period.—In the production of citric acid by the shallow-pan method, the fermentation is usually complete in 7 to 10 days

Yields.—On the basis of the sugar consumed, a maximum yield of 90 7 per cent of citric acid was obtained from glucose by Wells, Moyer, and May Carbon balances were prepared to show exactly how the carbon was used during the fermentations Clutterbuck and his associates have secured yields as high as 87 per cent on a semicommercial scale. Yields amounting to about 100 per cent on the basis of the sugar consumed have been reported by Butkewitsch and Gaewskaya. Usually, however, about 60 per cent of the weight of the sugar used in the medium may be recovered as citric acid.

Recovery of Citric Acid.—At the completion of the fermentation, the solution is drained off and the mat is pressed to remove any acid contained in it Calcium citrate is then precipitated from a hot neutral solution By treating the precipitate with an equivalent of sulphuric acid, the citric acid is liberated and is recovered by separating it from the calcium sulphate

The unconverted sugar may be fermented by yeasts and the citric acid crystallized directly, in an alternate method

Cultural Methods.—The successive transfer of spores from one lot of a medium to another of the same uniform composition may stimulate the mold to produce large yields of citric acid. Doelger and Prescott[1] have demonstrated the effect of 18 successive transfers on titrable acidity. Twelve 250-cc. flasks, each containing 75 cc. of the standard medium (14 per cent sucrose) adjusted to a pH of 2.00, were inoculated with the spores of a strain of *Aspergillus niger* from a single culture and incubated at a temperature of 26°C. for 10 days. Twelve new flasks were inoculated

TABLE 124.—AVERAGE TITRABLE ACIDITY PRODUCED WITH EIGHTEEN SUCCESSIVE SPORE TRANSFERS

No. of inoculation series	Average titrable acidity, normality	Increase in titrable acidity from low point, normality	Average spores on mat, per cent
1	0 4544	......	10 1
2	0 3438	......	53 2
3	0 2844	0 0000	32.7
4	0 3298	0.0454	14.5
5	0 3430	0.0586	9.5
6	0 3780	0.0936	8 0
7	0.3250	0.0406	6.7
8	0 4703	0.1859	5 0
9	0 4308	0 1464	5 5
10	0 4801	0.1956	7.1
11	0 3595	0 0751	32 2
12	0 5507	0 2663	3 3
13	0 5213	0.2369	3 1
14	0 5718	0 2774	3 3
15	0.4717	0 1873	1 4
16	0 6959	0 4115	1 6
17	0 6232	0.3388	3 3
18	0 8116	0 5272	1.3

from the spores that appeared on the mycelial mats of the first set of flasks This process was repeated at intervals of 10 days for about 8 months Results of this experiment are shown in Table 124.

The figures contained in the preceding table indicate a general increase in the level of titrable acidity with successive transfers. The drop in titrable acidity after the first three transfers may be ascribed to the fact that the mold had been grown previously in a 10 per cent sucrose solution and required time to become adjusted to the new concentration. After producing a titrable acidity approximately equivalent to a 1.2 N solution, there is usually no further increase in the titrable acidity.

[1] DOELGER and PRESCOTT, *loc cit.*

The right-hand column of the table shows the relation of sporulation to yields. The term "average spores on mat" refers to the approximate percentage of the surface covered by spores. Those fermentations in which high yields of citric acid were obtained showed only a few spores or even a complete absence of spores. Thus sporulation may be used as a means of judging the efficiency of a fermentation

By seeding only one-fourth to one-half of the surface area of the medium with spores, Doelger and Prescott obtained the best results. Uniform sprouting of the spores is prevented if the entire surface is seeded, with the result that unsprouted spores become embedded in the mycelial mat. It is believed that the unsprouted spores may exert a toxic effect toward citric acid production

For a detailed discussion of the technique of the fermentation, the reader is referred to the publications of Currie, Doelger and Prescott, and others (see the bibliography at the end of the chapter).

Characteristics of the Fermentation.—Sterile nutrient sugar solutions in shallow pans are inoculated with mold spores and incubated at the most favorable temperature for fermentation. The spores commence to sprout after a few hours and within 2 to 5 days the surface of the medium is covered by a firm, mycelial mat. With the formation of the mycelial felt, citric acid production proceeds at a rapid rate, and the fermentation is usually completed in 7 to 10 days.

Figure 79[1] shows curves for titrable acidity, pH, and weight of the mat for mashes that were allowed to incubate for 20 days at 24°C.

When about 90 per cent of the sucrose originally present in the medium has been converted to other products, the rate of increase of titrable acidity diminishes. Likewise when the concentration of citric acid is greater than approximately 7 per cent, it retards the rate of increase of titrable acidity. In a normal fermentation, the titrable acidity increases up to the ninth or tenth day, at which time there will be 7 to 8 per cent of citric acid and less than 1 per cent of oxalic acid. (An 8 per cent citric acid concentration is equivalent to a 1.2 N solution.) Citric acid is broken down unless it is recovered within a reasonable time after being produced

Production from Cane Molasses.—Perlman, Kita, and Peterson[2] studied citric acid production from cane molasses They found that yields by strains of *A niger* from untreated solutions of molasses were low in comparison with those obtained from synthetic and beet-molasses media The synthetic medium contained 140 g. of purified sucrose, 2.25

[2] Perlman, D, D A Kita, and W H Peterson, *Arch. Biochem*, 11 (No 1): 123 (1946)

g. of NH_4NO_3, 1.00 g. of KH_2PO_4, 0.25 g. of $MgSO_4 \cdot 7H_2O$, 0.1 mg. of Fe (as $FeNH_4(SO_4)_2 \cdot 12H_2O$), HCl to a pH of 2.3, and distilled water to 1 liter. The beet-molasses medium contained 280 g. of beet molasses diluted to 1 liter with distilled water. This was treated by adding 12 g. of $K_4Fe(CN)_6 \cdot 4H_2O$, 2 ml. of 1 N H_2SO_4, and 10 g. of filter cell, and
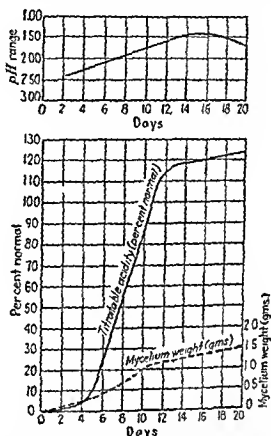
Fig. 79—Rate of increase in titrable acidity, weight of mat, and pH range in flask fermentations. [From W. P. Doelger and S. C. Prescott, Ind. Eng Chem., 26: 1142 (1934)]

permitting precipitation to take place over a period of 2 days at 10°C. The supernatant solution had a pH of 6.5.

Replacement of a part of the synthetic, or beet-molasses, medium with a Cuban high-test molasses medium (180 g. of molasses, 2.25 g. of NH_4NO_3, 1.00 g. of KH_2PO_4, 0.25 g. of $MgSO_4 \cdot 7H_2O$, and distilled water to 1 liter), or the addition of small amounts of ash from the Cuban high-test molasses to these media, resulted in lower yields of citric acid. The ashes from other samples of cane molasses and from untreated beet-molasses gave similar results. However, the ash from ferrocyanide-treated beet molasses did not cause a reduction in yields when added to the synthetic and beet-molasses media. Thus, Perlman and his associates concluded that the inorganic materials found in molasses were responsible for at least part of the inhibitory effect on citric acid production.

Treatment of the molasses with cation exchange material or with potassium ferrocyanide reduced the amount of ash and resulted in improved yields in most cases

Lamb;[1] Das Gupta, Saha, and Guha,[2] and Chatterjee[3] also have reported on studies made on the use of molasses for citric acid production.

Production from Beet Molasses.—The production of citric acid from beet molasses has been investigated by Roberts and Murphy,[4] by Gerhardt, Dorrell, and Baldwin,[5] and by others.

In the laboratory method of Roberts and Murphy[4] beet molasses containing calcium carbonate is impregnated on sphagnum moss The soaked sphagnum moss is distributed in a layer 1 to 2 in. deep, inoculated with the spores of *A. niger*, and incubated at 25°C Yields of 25 to 30 g. of calcium citrate from 200 g. of molasses have been obtained in 2 days. Calcium gluconate is also produced

The molds used by Gerhardt and associates were two strains of *A. niger*: strain 62 (No 67 of Wells, Moyer, and May—1936), and strain 72 (American Type Culture Collection No. 1015). These were selected from a total of 20 on the basis of their superior fermentation ability. Spores for inoculation purposes were grown in bottle plates containing sucrose nutrient agar. After 6 to 25 days, spore suspensions were prepared from the cultures and were standardized to contain about 50,000,000 spores per ml . Approximately 1 ml. of suspension was used to inoculate a surface area of 50 cm ^{2} and 20 ml for 5 liters of medium (5 cm. deep) in an aluminum pan measuring $36 \times 25.5 \times 7.5$ cm.

Although four samples of Straighthouse beet molasses from different localities were treated with potassium ferrocyanide, optimum conditions, which were as follows, were determined for only one sample: A quantity of 340 g. of beet molasses, partially diluted with water, was treated with 0 60 g of potassium ferrocyanide (in solution) The mixture was made up to 1 liter with distilled water Ten grams of diatomaceous earth were then added and mixed in uniformly The mixture was permitted to stand overnight at about 6°C in a graduated cylinder or similar container The treated medium was dispensed in the fermentation containers (6-oz bottles, glass tumblers, or shallow aluminum pans) and

[1] Lamb, A R , *Proc Hawaiian Sugar Planters' Assoc , Rept Expt Sta* , **56** : 142–143 (1936)

[2] Das Gupta, G C , K C Saha, and B C Guha, *Jour Indian Chem Soc , Ind & News Ed* , **3** : 64 (1940)

[3] Chatterjee, N P , *Jour Indian Chem Soc., Ind & News Ed.*, **5** : 201 (1942).

[4] Roberts, O , and D Murphy, *Sci Proc Roy Dublin Soc* , **23** : 307 (1944).

[5] Gerhardt, P., W W Dorrell, and I L Baldwin, *Jour Bact* , **52** (No 5), 555 (1946)

autoclaved at 120°C. for 15 min. The sugar concentration of the final medium was about 15 per cent.

Effective treatment of beet molasses with potassium ferrocyanide, based on the yields of citric acid subsequently obtained from the medium, was found to depend upon the concentration of ferrocyanide used, the pH of the molasses, and upon the conditions of sterilization. Relatively small variations from the optimum concentration of potassium ferrocyanide were found to result in reduced yields from the one sample of molasses tested. However, the yields of citric acid obtained from beet molasses treated with potassium ferricyanide were nearly equal, and small variations from the optimum concentrations did not appreciably influence the yields. The optimum pH for the ferrocyanide treatment of beet molasses was about 7, regardless of whether the adjustment of pH was made before or after precipitation or after sterilization. Adjustment of the pH was unnecessary in the case of some samples of beet molasses The yields of citric acid were higher for one sample of beet molasses when the medium was sterilized, and for three other samples when the medium was not sterilized.

The media were incubated at 30°C. for 10 days after inoculation.

The yields of citric acid obtained from beet molasses in the shallow aluminum pans (covered loosely during fermentation with sheet aluminum) were 45 to 50 per cent based on the available sugar.

Citric Acid by the Cahn Method.—A rather unusual method of citric acid production was advocated by Cahn[1] in 1934. Solid material, such as cane or beet pulp, is impregnated with sugar solutions—sucrose or molasses. The unsterilized mass is inoculated with a mold culture, the age of which is said to be relatively unimportant, and fermentation proceeds rapidly over the relatively large surface at a temperature of 20 to 35°C. The fermentation is usually complete in 4 days or less, at which time a yield of 55 per cent citric acid on the basis of the sucrose originally present, or 45 per cent acid, calculated on the basis of the sugar originally present in the molasses, may be expected. One pound of citric acid may be secured from 5.7 lb. of molasses and 2.75 lb. of beet pulp (beet pulp may be used only once, while cane pulp may be used more than once).

Production of Citric Acid by a Mucor.[2]—Solutions of molasses and gur have been fermented by a species of *Mucor* with the production of citric and oxalic acids. By controlling the conditions of the fermentation, citric acid may be obtained with little or no oxalic acid. A molasses mash containing 1 per cent added ammonium sulphate, adjusted to a pH of 4,

[1] CAHN, F J , *Ind Chem.*, **27:** 201 (1935).

[2] DAS GUPTA, G. C , SAHA, K. C., and B. C. GUHA, *Science and Culture* **3** (No 7): 397 (1938).

and incubated at a temperature of 28°C. for a period of 7 days yielded 33 per cent of citric acid on the basis of the sugar.

The Production of Citric Acid on an Industrial Scale.—The earliest attempt to produce citric acid by fermentation on an industrial scale was made in Germany around the beginning of the twentieth century. The many difficulties encountered made it impossible to meet the competition offered by the citrus-fruit industry and the attempt was abandoned.

It is believed that some citric acid was produced by fermentation on a commercial basis in the United States in the year 1919, but it was not until the year 1923 that appreciable quantities were manufactured by the mycological process, the first factory in the world for the commercial production of citric acid by fermentation being erected in New York that year. During the year 1929,[1] about 7,000,000 lb of citric acid were produced by mold fermentation in this country, while at present over 20,-000,000 lb. are being manufactured annually by this method. The industry has expanded so rapidly that the United States has not only become independent of outside sources but, for a number of years, has exported large quantities of calcium citrate, principally to England. These exports have dropped markedly since 1935,[1] because England has developed her own fermentation process and the domestic demand for citric acid has become greater.

England, Belgium, Czechoslovakia, and, probably, Russia now produce large amounts of citric acid by fermentation, following the successful establishment of the industry in the United States

Although the details of the commercial production of citric acid have not been made public, it is believed that the acid is produced in shallow pans, using a strain of *Aspergillus niger*.

Production of Citric Acid by Submerged-culture Methods.—Early efforts to produce citric acid by submerged-culture methods resulted in failure   Yields produced by this method were lower than those obtained by surface-culture methods, and occasionally gluconic acid was produced at the expense of citric acid

Wehmer[2] (1912) endeavored to produce citric acid from a nutrient sucrose medium containing chalk by passing sterile air through it. Citric acid was produced as calcium citrate, but the conversion was low. Similar results were obtained by Elving,[3] who caused the mold to grow in submerged cultures by means of aeration   In 1926, Bleyer[4] reported on a process in which citric acid was produced in vats supplied with air and

[1] Wells, P A, and H T Herrick, *Ind Eng Chem.*, **30**: 255 (1938).
[2] Wehmer, C , *Chem Ztg*, **36**: 1106 (1912).
[3] Elving, F , *Soc. Forh Math Nature* , **18** (1918-1919)
[4] Bleyer, B , German Patent 431729, Oct. 6, 1926

occasional mechanical agitation. Schreyer[1] discovered that aeration and continuous mechanical agitation increased the total production of acid by *A. fumaricus* over that of controls in which the surface culture method was used, but that gluconic acid and not citric acid production was increased. Results obtained by Thies[2] were similar to those of Schreyer, for he found that aeration of the medium with sterile oxygen increased gluconic but not citric acid production. In 1930, Amelung[3] investigated the production of citric acid from sucrose by *A. japonicus* in submerged culture using aeration. Although good yields were obtained by this method, they were not as high as those obtained by the older method of culture in shallow layers without aeration. Perquin[4] discovered that the addition of solutions of glucose or sucrose to the preformed mycelium of *A. niger* resulted in gluconic acid production. However, some citric acid was produced when the solution contained zinc sulphate, potassium chloride, and ammonium chloride and was at a low pH.

Successful production of citric acid on a laboratory or pilot-plant scale has been reported by Perquin,[4] Karow,[5] Szücs,[6] Waksman and Karow,[7] Waksman,[8] Karow and Waksman,[9] Shu and Johnson,[10,11] Perlman,[11] and others.

Some Factors Affecting Production.—There are a number of factors that affect citric acid production by submerged-culture methods. Some of these will be discussed now, others later.

Strains of molds differ markedly in their ability to produce citric acid by the submerged-culture method. Perlman[13] confirmed this by testing 70 strains of *A. niger* for their ability to produce citric acid on five different media, glucose salts, sucrose salts, maltose syrup, beet molasses, and ferrocyanide-treated beet-molasses media. The facts he derived emphasize the need for selecting strains carefully.

[1] SCHREYER, R., *Biochem. Zeit.*, 202: 131 (1928)

[2] THIES, W., *Zentr. Bakt. Parasitenk*, II. 82: 321 (1930)

[3] AMELUNG, A., *Chem Ztg*, 54: 118 (1930)

[4] PERQUIN, L. H. C., Doctor's Thesis, Delft, 1938.

[5] KAROW, E O, Doctor's Thesis, New Brunswick, N.J., 1942.

[6] SZUCS, J., U S Patent 2,353,771, July 18, 1944

[7] WAKSMAN, S. A., and E O KAROW, U S Patent 2,394,031, 1946

[8] WAKSMAN, S. A., U.S. Patent 2,400,143, May 14, 1946

[9] KAROW, E O, and S. A. WAKSMAN, *Ind. Eng. Chem*, 39: 821 (1947).

[10] SHU, P, and M. J JOHNSON, Paper presented at meeting of A. C. S. at Atlantic City, April, 1947.

[11] SHU, P, and M. J. JOHNSON, *Ind Eng. Chem.*, 40 (No. 7): 1202 (1948).

[12] PERLMAN, D, Paper presented at Fourth International Congress of Microbiology.

[13] PERLMAN, *op. cit.*

He reported that young spores are better than old ones for producing citric acid and that the temperature of storage of spores (on agar slants) has a big effect on their ability to produce citric acid. Of the temperatures tested, 0 to 29°C., the range of 0 to 5°C is best for storage. Deterioration in ability to produce citric acid is most rapid at 29°C

The influence of the composition of the sporulation medium on citric acid production by *A niger* (strain 72–4) has been studied by Shu and Johnson.[1] They made a number of significant observations and conclusions The effect of *increasing the concentrations of zinc, ammonium nitrate, and potassium dihydrogen phosphate in the basic sporulation medium was to decrease the rate of spore formation.* Increasing the concentrations of manganese to 9 3 mg./liter and malt extract to 1.5 g /l favored abundant spore formation within 48 hr. instead of the usual 68 hr

However, the addition of 9.3 mg per liter of manganese to the sporulation medium reduced the yield of citric acid from the fermentation medium, whether used alone or in combination with traces of zinc, copper, and/or iron. It was shown that the amount of manganese carried from the sporulation medium with washed spores was sufficient in amount to reduce the yields of citric acid from the fermentation medium, and that a mere 3 micrograms of manganese per liter in the latter medium lowered yields appreciably.

Trommer malt extract, but not its ash, when added to the sporulation medium reduced citric acid production in submerged cultures. Neither the malt extract nor manganese had any appreciable effect on citric acid production by the surface culture method, however.

Perlman[2] also observed that when zinc and/or manganese salts were added to a purified agar-sucrose-salts medium, the spores produced thereon often produced low yields of citric acid. However, this varied considerably with the strain of mold used

Temperature of incubation plays an important part in the production and accumulation of citric acid As a rule most strains produce considerably more citric acid at 26 to 30°C. than at 39°C Perlman also showed that there may be a loss in the citric acid content of flasks if these are placed at a higher temperature following initial incubation at a lower temperature.

The influence of heavy metals on surface culture fermentations has already been referred to Such ions also have an effect on citric acid production by the submerged culture method, depending on the strain of mold used and other factors There appears to be an optimum concentration for each ion The low yields of citric acid obtained from some

[1] Shu, P , and M J Johnson, *Jour Bact*, **54** (No. 2)· 161 (1947)

[2] Perlman, *op cit*

technical grades of sugar and molasses are undoubtedly due, in part at least, to an inhibiting concentration of certain ions. Sjolander[1] has shown that the addition of the ash of these carbohydrate sources to a synthetic medium will result in a reduced yield of citric acid.

Production with Aspergillus wentii.—This fermentation has been described in a series of publications by Karow and Waksman.[2-5] The following discussion is based on these reports.

The process used by Karow and Waksman is singular in several respects: (1) the mold, (2) the use of a special medium for the growth and conditioning of the mold, (3) the aeration of the medium with oxygen or air under pressure together with mechanical agitation, (4) the use of a replacement medium for the production of citric acid with preformed cell material, and (5) the partial neutralization of the citric acid in the replacement medium to increase the yield of citric acid. Production by this method may be divided into 2 phases—a growth and a fermentation phase.

THE MOLD.—A selected strain of *Aspergillus wentii* produced the highest yields of citric acid by submerged growth in the researches carried out by Karow and Waksman. *A. niger* 2.3, which was used by Perquin, was found to be inferior to *A. wentii*.

The colonies produced from the mold spores of *A. wentii* under submerged-growth conditions are generally globular in appearance but occasionally may be stringy. Superior results in the production of citric acid have been achieved through the use of cultures containing globular colonies. Stringy colonies usually result from too rapid growth brought about by the inclusion in the medium of substances that are particularly favorable to growth, such as high nitrogen and iron.

CARBOHYDRATE SOURCES.—Various sources, such as sucrose, glucose, corn sirup, Hydrol, sugar cane juice, raw sugar, and molasses, are satisfactory for citric acid production. Sucrose, in particular, and glucose are good sources. Raw sugar, though good, is inferior to sucrose. Purified cane molasses is suitable, the yields from some types being as high as those obtained from pure cane sugar.

Ion exchange resins, activated carbon, bone char, and bauxite may be used to purify the molasses. The treatment of molasses with a combination of bone char, Suchar CSP, and Amberlites (ion exchange resins) yielded excellent results.

[1] SJOLANDER, J R , *Bachelor of Science Thesis*, University of Wisconsin, 1945
[2] KAROW, *op cit*
[3] WAKSMAN, and KAROW, *op. cit.*
[4] WAKSMAN, *op. cit.*
[5] KAROW and WAKSMAN, *loc. cit.*

THE GROWTH MEDIUM.—Karow found that the following medium was best for growing *A. wentii* and citric acid production:

Sucrose....	150 g
Urea........	1 0 g
MgSO₄.7H₂O	0 5 g
KH₂PO₄....	0 08 g.
KCl............	0 15 g
MnSO₄.4H₂O	0 02 g
ZnSO₄.7H₂O.	0 01 g
Water (distilled) to .	1,000 ml.
pH adjusted to 2 0 with HCl	

The use of higher concentrations of urea resulted in increased growth. A high nitrogen content in general increases growth and the consumption of sugar, but tends to decrease the amount of citric acid produced

The amount of magnesium sulphate used may be varied appreciably without seriously affecting growth or citric acid production, as long as sufficient is furnished to supply basal requirements Effects due to the use of larger quantities of magnesium sulphate usually appeared early in the fermentation and disappeared later.

The optimum concentration of manganese sulphate appeared to be 20 mg per liter. Larger concentrations of this salt did not stimulate citric acid production. It is possible that the manganese may act by helping to maintain a favorable oxidation-reduction potential.

Zinc sulphate in the proportion of 10 mg. per liter increased the yields of citric acid when manganese sulphate was also present in a concentration of 20 mg. per liter. Without manganese sulphate, zinc sulphate decreased the yields of citric acid. Manganese thus tends to antagonize the inhibitory effects of zinc.

Other effects of salts on citric acid production by *A. wentii* have been found. For example, the omission of potassium chloride from the basal medium appeared to result in decreased acid production.

Accessory growth factors had no apparent effect on the production of citric acid by *A. wentii*. Thiamin chloride, pantothenic acid, riboflavin, pyridoxine, nicotinic acid, ascorbic acid, and *p*-aminobenzoic acid, each alone, and in combination, were used in the basal medium without effect on citric acid production. Corn steep liquor increased the rate and amount of growth and total titrable acidity but not the citric acid yields Yeast extract also increased the rate and amount of mycelial growth, and sugar consumption, but did not produce appreciable increases in citric acid accumulation.

INOCULATION OF THE MEDIUM.—Karow states that citric acid production by *A. wentii* from the growth medium is inversely proportional to the

size of the inoculum.   For 100-ml. amounts of medium, an inoculum containing 0.25 to 1.0 ml. of spores in suspension is adequate. *Amounts larger than 1 ml. result in lower yields of citric acid.*

After the medium is inoculated it should be permitted to stand for 12 to 18 hr. before agitation and oxygenation are commenced, otherwise the yield of citric acid is decreased.

EFFECT OF OXYGEN.—Oxygen has a profound effect on the production of citric acid by *A. wentii.* Best yields are obtained when oxygen is passed through the submerged cultures. Aerated cultures produce larger yields of citric acid than unaerated cultures, but yields from aerated cultures are decidedly lower than those from oxygenated cultures. *The use of oxygen results in greater sugar consumption and development of mycelium. Oxygen, pure or combined with nitrogen, is essential for the production of citric acid by A. wentii, also for the growth of the mold in submerged cultures*

EFFECT OF CARBON DIOXIDE.—Although it has been shown by Foster, Carson, Ruben, and Kamen[1] that carbon dioxide participates in the *synthesis of citric acid by A. niger, the addition of this gas to submerged cultures of A. wentii at pH 2 0 does not result in increased yields of citric acid.* Actually 5 and 15 per cent concentrations of carbon dioxide inhibit the production of this acid The supply of carbon dioxide also results in a lower consumption of sugar and decreased growth of cell material.

THE REPLACEMENT MEDIUM.—The replacement medium was devised in order to study the effect of various minerals on preformed cell material. The latter was produced by inoculating a sterile nutrient *solution* (described above) with a heavy spore suspension of *A. wentii,* incubating at 28°C , and aerating with sterile air. *Cell material available after 4 days' incubation was used in the study, since it was discovered that young mycelium was more efficient than old.*

The replacement medium, selected after considerable research, had the following composition ·

Sucrose	150 g
Urea	0 5 g
KH₂PO₄	0 05 g
KCl	0 15 g
MnSO₄ 4H₂O	0 02 g
ZnSO₄ 7H₂O	0 01 g
Water to	1,000 ml

Sufficient nutrient salts were added to the medium to maintain the mold in an active enzymic condition and to produce a slight increase in growth

[1] FOSTER, J W , S F CARSON, S RUBEN, and M D. KAMEN, *Proc. Nat Acad Sci ,* 27: 590 (1941)

Nitrogen (from urea) and **phosphate (from KH$_2$PO$_4$)** stimulated the production of citric acid. Magnesium sulphate had an inhibiting effect and thus was not added to the medium.

EFFECT OF PARTIAL NEUTRALIZATION OF REPLACEMENT MEDIUM — Partial neutralization of the acid produced by *A wentu* in the replacement medium by the use of calcium carbonate leads to increased production of citric acid. When the acid is completely neutralized, oxalic rather than citric acid is formed. Neutralization of the acid to a pH higher than the range of 3 0 to 3 5 leads to oxalic acid production, as does neutralization with sodium, potassium, or ammonium hydroxide As much as a third of the acid can be neutralized with CaCO$_3$ without producing a pH as high as 3 5

PILOT-PLANT SCALE PRODUCTION.—Karow carried out an experiment using a large bottle. Five liters of the growth medium were placed in it and inoculated with the spores of *A wentu* The bottle was caused to rotate on its side at the rate of 21 r p m and 6 to 8 liters of oxygen per hour were supplied to the medium After the tenth day, acid production dropped sharply The cell material was filtered, and washed with sterile distilled water. Five liters of the replacement medium were added, and fermentation continued for 10 days under conditions similar to the preceding From the 4 liters of the growth solution, 135 6 g of anhydrous citric acid were recovered; from 3 7 liters of the replacement solution, 198 7 g

For further details on this process, the reader is referred to the reports by Karow and Waksman cited herein

Production by the Szucs Method.[1]—In this method for producing citric acid by submerged fermentation, the mold is first cultured in a growth solution. The latter may be inoculated with the spores, pregerminated spores, mycelium, or comminuted mycelial mat of *A. niger* or other mold species that has the ability to produce citric acid from carbohydrates by surface fermentation. Finely dispersed, sterile, oxygen-containing gas is passed through the medium, which is also stirred After developing sufficiently in the growth solution, the mycelium is separated out by centrifuging, decanting, or filtration, and placed in a second solution, known as the fermentation solution This solution contains a carbohydrate, such as sucrose, fructose, glucose, purified molasses, corn sirup or corn sugar, and nutrient salts, but contains no assimilable phosphorus compounds. (The presence of a small quantity of phosphorus compounds will retard but not prevent the formation of citric acid) The fermentation solution is agitated and stirred An oxygen-containing gas (unmixed oxygen, or mixtures of oxygen with nitrogen or air) is passed

[1] Szücs, *op cit*

through the solution in a finely dispersed state, with or without pressure. For example, oxygen or a mixture of oxygen and air containing 40 to 50 per cent by volume of oxygen, may be passed through the solution at the usual atmospheric pressure or under pressures of 1 to 4 atmospheres. Active carbon, ascorbic acid, glutathione, or other accelerators that act as oxygen carriers, may be added to the solution. Calcium carbonate or some other suitable neutralizing agent may be added to the fermentation solution before or during the fermentation for the purpose of neutralizing the citric acid. The fermentation may be carried out in the temperature range of 18 to 28°C. However, a temperature of 25°C is preferred by Szücs. After the fermentation is completed, the mycelium may be removed from the solution and used in one or more additional submerged fermentations.

An example of this fermentation follows:[1] A growth solution, which contained 25 to 50 g. of sucrose, 2.25 g. of NH_4NO_3, 0.3 g. of KH_2PO_4, 0.25 g. of $MgSO_4 \cdot 7H_2O$, and 10.0 ml. of N/1 HCl (pH 2.0) in 1 liter, was sterilized and inoculated with the spores of *A. niger* in aqueous suspension. The solution was stirred and sterile air in a finely dispersed condition was passed through it. After growth for 3 to 4 days at 25°C, the mycelium was separated from the growth solution and washed with distilled water. The washed mycelium (5 parts by weight in terms of dry mycelium for 1,000 parts by weight of the fermentation solution) was then placed into the fermentation solution, which contained 200 g. of sucrose, 1.1 g. of NH_4NO_3, 0.15 g. of KCl, 0.25 g. of $MgSO_4 \cdot 7H_2O$, and 10 ml. of N/1 HCl (pH 1.91) in 1 liter. The fermeatation solution was agitated vigorously with a rotary stirrer revolving at 300 r.p.m and finely dispersed oxygen was passed through it (100 ml. through 2 liters of solution per minute). The temperature was maintained at 25°C. In about 4 days, a yield of 70 to 75 per cent of citric acid, on the basis of the sugar consumed by the mold, was secured.

Production by the Method of Shu and Johnson.—According to Shu and Johnson,[2,3] yields of 72 g. of anhydrous citric acid may be obtained from 100 g. of sucrose in 9 days by a submerged fermentation process, while using a strain of *A. niger* derived from A.T.C.C. No 1015.

The organism was carried in soil cultures. Before use it was transferred twice on sugar-agar slants and then cultured on a sporulation medium (medium A in Table 125) for 3 to 5 days. The suspension prepared from the spores was used as the inoculum.

[1] *Ibid.*

[2] Shu, P., and M J. Johnson, Paper presented at meeting of A C S. at Atlantic City, April, 1947.

[3] Shu, P, and M J. Johnson, *Ind. Eng. Chem.*, *loc. cit.*

The basal medium was prepared as indicated in Table 125, adjusted to a pH of 3 8 with hydrochloric acid and distributed in 50-ml. amounts in 500-ml. Erlenmeyer flasks. These were inoculated with spore suspensions. The flasks were then agitated at 270 r p m on a rotary shaker in such a manner that each described a horizontal circle 1 in in diameter. The incubation temperature was 25°C.

A number of factors were found to be important in obtaining good yields of citric acid. Sucrose concentrations of 140 to 264 g per liter were

TABLE 125 —COMPOSITION OF MEDIA[1]

Constituents	Medium A, wt /liter	Medium B, wt /liter
Domino sucrose, g	140	140
Bacto agar, g	20	
KH₂PO₄, g	1 0	2 5
MgSO₄·7H₂O, g	0 25	0 25
NH₄NO₃, g	2 5	2 5
HCl .		To pH 3 8
Trace metals[2]		
Cu⁺⁺, mg	0 48	0 06
Zn⁺⁺, mg	3 8	0 25
Fe⁺⁺⁺, mg	2 2	1 3
Mn⁺⁺, mg[3]	< 1 0	< t 0

[1] SHU, P , and M. J. JOHNSON, Eng Chem , 40 (No 7) 1202 (1948)
[2] Listed quantities of metals include amounts present as impurities in other constituents of the medium. Media were sterilized at 120°C for 15 min
[3] The importance of low manganese concentrations in both sporulation and fermentation media for submerged citric acid production is demonstrated in work reported elsewhere.

optimum for citric acid production on the basis of sugar utilized Low yields were obtained with low sugar concentrations It was necessary to keep the manganese content of both the sporulation and fermentation media low to obtain good yields.[1] Magnesium sulphate in concentrations below 0 5 g per liter resulted in lower yields, while amounts from 0 5 to 2 g per liter did not affect the conversion efficiency Iron was involved directly with the growth of the mycelium and with the citric acid yield. A concentration of 1 mg per liter was optimum At too low concentrations of iron the utilization of sugar was poor Large concentrations favored heavy growth of the mycelium which was inclined to result in smaller yields. The phosphate ion appeared to be concerned with citric acid production, in addition to its effects as a buffer and food constituent The optimum initial pH range was 2.2 to 4 2. At too low initial pH levels, growth and acid production were retarded Maximum yields were

[1] SHU, P., and M. J. JOHNSON, Jour. Bact , loc cit

obtained in 8 days with the aeration and agitation provided by a rotary shaker and in 11 days by a reciprocating type of shaker.

The chemical changes occurring during a fermentation of the basal medium (medium B in Table 125), containing 25 per cent sugar and at an initial pH of 4.5, are shown in Fig. 80.
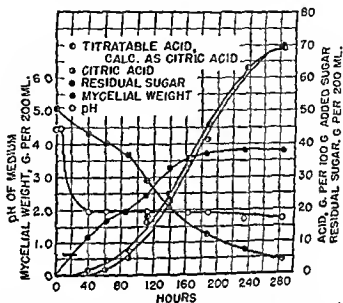
Fig. 80—Chemical changes during fermentation. [Courtesy of P. Shu and M. J. Johnson, Ind. Eng. Chem., 40 (No. 7), 1202 (1948).]

Effect of Treatment of Commercial Sugars with Ion Exchange Agents.—Perlman, Dorrell, and Johnson[1] found that commercial grades of sucrose and glucose gave low yields of citric acid when fermented by A. niger 62, due to the metallic ions contained. However, after the sugars had been treated by passage over cationic exchange materials, such as "Alkalex" (Research Products Corp., Madison, Wis.), "Amberlite IR-100" (Resinous Products & Chemicals Co., Philadelphia), and "Zeo-Karb H" (Permutit Co., New York), the yields were increased more than threefold (refer to Table 126).

Uses.—Approximately 65 per cent[2] of the citric acid used in the United States is employed for medicinal purposes (citrates, etc.), 15 per cent in foods (flavoring extracts, soft drinks, etc.), 9 per cent in candies, and small quantities as an ingredient of ink, in silvering, in dyeing and calico printing, and in engraving, according to the U.S. Department of Commerce.

Theories Concerning the Mechanism of Citric Acid Formation.—Although many theories have been advanced to explain the formation of

[1] Perlman, D., W. W. Dorrell, and M. J. Johnson, Arch. Biochem., 10 (No. 3), 131 (1946).
[2] Ibid.

citric acid from carbon-containing compounds, none proposed has satisfactorily explained all the known observations. Various theories are here presented to show the complexity of the reactions that may occur in this fermentation rather than to assume that any one of them shows the true course. Any theory proposed must account for the production of citric

TABLE 126.—EFFECT OF TREATMENT OF COMMERCIAL SUGARS BY IONIC EXCHANGE METHOD ON ACID FORMATION (STRAIN 62)[1]

Sugar	Treatment	Yield of citric acid, per cent	Yield of oxalic acid, per cent	Sugar fermented, per cent	Length of fermentation, days
Cane sucrose	None	21 4*		31.4	8
	Cationic exchange	61 0	4 1	98 0	8
Beet sucrose	None	11 3*		17 6	8
	Cationic exchange	66 8	5 6	97 5	10
Glucose[2]	None	20 5*		33 2	8
	Cationic exchange	65 0	7 2	99 0	10

[1] PERLMAN, D W W, DORRELL, and M J JOHNSON, *Arch Biochem*, 10 (No 3), 131 (1946) Salts indicated in medium A were used Yields reported are for day of maximum yield of citric acid.
* Cerelose* (glucose monohydrate)
* Titratable acidity calculated as citric acid

acid from 2-, 3-, 4-, 5-, 6-, 7-, and 12-carbon compounds if it is to be acceptable It must account for high yields of citric acid from sugars, which in some instances have approached 100 per cent on the basis of the sugar consumed

Maze and Perrier (1904) suggested that citric acid arose as a product of incomplete respiratory metabolism.

Parasaccharinic acid played an important part as an intermediate in a theory proposed by Buchner and Wustenfeld Sugar was broken down to parasaccharinic acid, which was rearranged to form citric acid. A condensation reaction would be necessary to explain citric acid formation from some sugars (Herzog and Polotsky, 1909).

Euler (1909) and others suggested that pyruvic acid was formed from sugar and that this was decomposed to acetaldehyde and carbon dioxide Three molecules of acetaldehyde condensed and the product was oxidized to citric acid

$$3C_3H_4O_3 \rightarrow 6CH_3CO \cdot COOH + 6CO_2 + 2C_3H_6O_3,$$
$$\text{Glucose} \qquad \text{Pyruvic acid} \qquad \qquad \text{Citric acid}$$
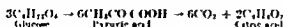
The maximum yield by this theory could only be 71 4 per cent of citric acid Therefore this hypothesis can be eliminated on quantitative grounds alone

In 1919, Raistrick and Clark suggested that hexose is broken down to

αγ-diketoadipic acid, which then becomes hydrolyzed to acetic and oxalacetic acid, these two acids combining to form citric acid.

| Glucose | Saccharic acid | αγ-Diketoadipic acid | Acetic acid + | Citric acid |
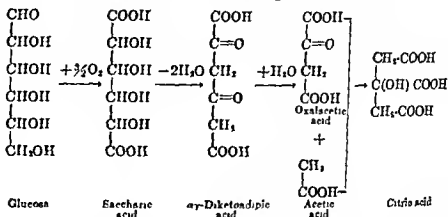
Oxalic acid may arise from the breakdown of oxalacetic acid and the oxidation of acetic acid:
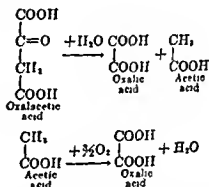
Although this theory can account for the high yields of citric acid from a hexose, it does not account for the production of this acid from substances other than a hexose, unless it is assumed that a hexose is synthesized in each case.

In 1924, Butkewitsch proposed a scheme in which glucose was directly oxidized to citric acid:

$$C_6H_{12}O_6 + \tfrac{3}{2}O_2 \rightarrow C_6H_8O_7 + 2H_2O$$
$$\text{Glucose} \qquad\qquad \text{Citric acid}$$
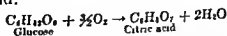
Butkewitsch was of the opinion that glucose passed first to gluconic acid, which was subsequently transformed to glucuronic acid. The latter was then supposed to undergo an intramolecular aldol condensation to form a five-membered ring. Rupture of the ring, followed by oxidation, led to the formation of citric acid:
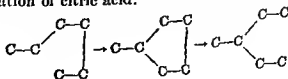
Wehmer (1925) suggested the possibility of the following reaction taking place, since calcium gluconate was fermented by *Aspergillus niger* to citric acid:

Glucose → Gluconic acid → Citric acid → Oxalic acid → Carbon dioxide
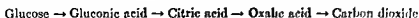
Saccharic acid was the principal intermediate product in a theory suggested by Franzen and Schmitt (1925):

$$\text{Sugar} \to \text{Saccharic acid} \to \beta\text{-}\gamma\text{-Diketoadipic acid} \xrightarrow[\text{acid rearrangement}]{\text{by benzine}} \text{Citric acid}$$

Challenger and his associates (1927) added weight to this theory, for they isolated saccharic acid from media containing glucose that had been fermented by *A. niger*. Calcium gluconate also yielded saccharic and citric acids. Citric acid was obtained from potassium hydrogen saccharate solutions inoculated with *A. niger*. Bernhauer, however, does not believe that saccharic acid is an intermediate product in the citric acid fermentation, as the result of his work with a large number of molds.

Theory of Chrzaszcz and Tiukow.—From the observations (1930) that certain molds have the ability to produce citric acid, along with succinic, fumaric, and malic acids, from acetic acid and ethyl alcohol, Chrzaszcz and Tiukow have formulated their scheme for the formation of these acids. Two molecules of acetic acid are dehydrogenated to 1 molecule of succinic acid, which, in turn, by loss of hydrogen, passes to fumaric acid. This acid is converted to malic acid upon the addition of 1 molecule of water. One molecule of malic acid and 1 molecule of acetic acid combine with the loss of hydrogen to form citric acid.
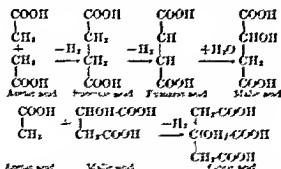
It was later suggested that the initial stage in the production of acid from sugars was similar to that of ethyl alcohol fermentation. If this fact were true, then the maximum yield of citric acid from glucose should not exceed 71.1 per cent and that of the weight of citric acid to carbon dioxide should not exceed 1.

shown definitely by the use of carbon balance experiments that yields considerably higher than 71.1 per cent may be obtained from glucose, also that the ratio 1.45:1 is greatly exceeded. These general observations have been corroborated by others.

Bernhauer's Theory.—Bernhauer, Böckl, and Siebenauger (1932) have suggested that acetic acid and ethyl alcohol are formed from sugars by molds in the manner indicated by Neuberg's scheme for alcohol production by yeasts. Acetic acid is converted to citric acid through aconitic acid as follows:

$$C_6H_{12}O_6 \xrightarrow[\substack{\text{Alcohol}\\\text{break-}\\\text{down}}]{} 2CH_3 \cdot COOH \xrightarrow{-H_2} \begin{array}{l} CH_2COOH \\ | \\ CH_2COOH \end{array} \xrightarrow{-H_2} \begin{array}{l} CH \cdot COOH \\ \| \\ CH \cdot COOH \end{array}$$

Glucose   Acetic acid   Succinic acid

$$\begin{array}{l} \text{Fumaric acid} \\ -H_2O\| +H_2O \\ CHOH \cdot COOH \\ | \\ CH_2 \cdot COOH \\ \text{Malic acid} \end{array}$$

$$\begin{array}{l} CH_2COOH \\ | \\ CH_2COOH \end{array} + CH_3COOH \xrightarrow{-H_2} \begin{array}{l} CH_2COOH \\ | \\ CH \cdot COOH \\ | \\ CH_2COOH \end{array} \xrightarrow{-H_2} \begin{array}{l} CH \cdot COOH \\ \| \\ C \cdot COOH \\ | \\ CH_2 \cdot COOH \end{array} \xrightarrow{+H_2O} \begin{array}{l} CH_2COOH \\ | \\ C(OH) \cdot COOH \\ | \\ CH_2 \cdot COOH \end{array} \xrightarrow{-H_2O} \begin{array}{l} CH_2COOH \\ | \\ C(OH) \cdot COOH \\ | \\ CH_2 COOH \end{array}$$

Succinic   Acetic   Aconitic   Citric
acid   acid   acid   acid

$$\begin{array}{l} | - CO_2 \\ CH_2 \\ | \\ C \cdot COOH \\ | \\ CH_2COOH \end{array}$$

Itaconic acid

The preceding scheme is based on several facts (see the theory of Chrzaszcz and Tiukow). Aconitic acid may be converted to citric acid by molds producing citric acid (one strain having formed over 20 per cent citric acid from aconitic acid) while citric acid may be transformed to aconitic acid. Methylglyoxal has been produced from sodium hexose-diphosphate by aspergilli, which have the ability to form citric acid.

This theory is not acceptable, however, because the maximum yield of citric acid by this scheme cannot exceed 71.1 per cent and the ratio of the weights of citric acid and carbon dioxide cannot exceed 1 45:1. Furthermore aconitic acid has not been isolated from fermentations of sugars by molds, although *Aspergillus itaconicus* produces itaconic, citric, and gluconic acids from sucrose and fructose.

Theory of Emde.—Emde's scheme (1935) for the conversion of sucrose to citric acid through quinic acid was proposed after it had been shown by Fischer and Dangschat that quinic acid could be oxidized to citric acid by periodic acid.

Quinic acid   Citric acid
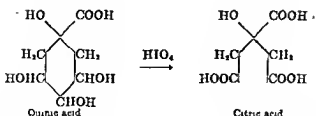
The maximum possible yield of citric acid from sucrose, according to the Emde's scheme, is 56 1 per cent.

$$C_{12}H_{22}O_{11} + 5O_2 \rightarrow C_6H_{12}O_6 + 5CO_2 + 5H_2O$$
Sucrose   Quinic acid
$$C_7H_{12}O_6 + \tfrac{1}{2}O_2 \rightarrow C_6H_8O_7 + CO_2 + 2H_2O$$
Quinic acid   Citric acid
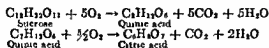
Therefore, on the basis of yields alone, this theory is untenable. Butkewitsch, who had suggested that only molds with the ability to produce citric acid were able to ferment quinic acid, was unable to obtain citric acid from quinic acid by fermentation.

Theory of Gudlet —The scheme proposed by Gudlet (1935) is based on the fact that glucose may split directly into a 4-carbon molecule, succinic acid, and a 2-carbon compound, acetaldehyde. (Virtanen originally suggested that this reaction might take place in the propionic acid fermentation.)
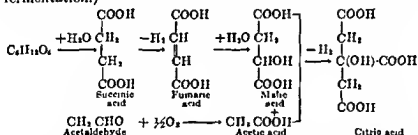
The high yields obtained from glucose, on the basis of the sugar consumed, could be explained by this scheme since decarboxylation is not concerned

The theory that acetic acid is directly converted to succinic acid and citric acid has been attacked by Butkewitsch, Menzshinskaya, and Trofimova (1935) who hold that citric acid is formed from substances of the mycelium by *Aspergillus niger*, the process being stimulated by the addition of acetic acid These investigators state that there is no direct connection between the consumption of acetic acid and the accumulation of citric acid, oxalic acid being the principal substance produced from the conversion of acetic acid.

By carefully weighing the mycelium, citric acid, acetic acid, and other products involved, Bernhauer and others have secured information

by which they have been able to refute the hypothesis of citric acid formation from substances of the mycelium.

Another hypothesis concerning the fermentation of acetic acid is that it is used in the synthesis of carbohydrates which are later broken down to citric or succinic acids. This theory has little evidence for support.

When citric acid is fermented anaerobically with yeast, each molecule of citric acid yields 2 molecules of acetic acid, 2 molecules of carbon dioxide, and some formic acid. *Salmonella acrtrycke* decomposes citric acid into acetic, succinic, and formic acids. These facts indicate a possible connection of citric acid with acetic, succinic, and other acids.

Theory of Ciusa and Brüll.[1]—Ciusa and Brüll have shown that the addition of malic acid, glycolic acid, or mixtures of the two acids to nutrient sugar solutions, adjusted to a pH of 3.5, increased the yields of citric acid produced from sugar by *A. niger*. The addition of malic acid increased the yield to 332 per cent of the sugar consumed, while glycolic acid increased the yield to 132 per cent. When equimolar quantities of malic and glycolic acid were added, the yield was increased to 928 per cent. On the basis of these results, Ciusa and Brüll suggested that the last phase of the intermediary reactions in the citric acid fermentation might be a condensation of malic and glycolic acids:
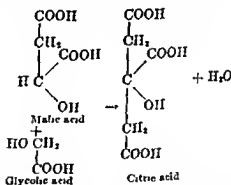
The citric acid fermentation might be schematically represented as follows, according to Ciusa and Brüll:
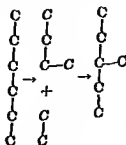
[1] Ciusa, R., e L. Brüll, *Ann. chim. applicata*, 29: 3–11 (1939).

For further details concerning the mechanism of the fermentation, consult some of the papers cited in the reference list.

References on Citric Acid and the Citric Acid Fermentation

AMELUNG, H : Beiträge zur Säurebildung durch *Aspergillus niger*, Zeit. physiol. Chem , 166: 161 (1927)

———. Wachstum und Säurebildung von *Aspergillus niger* unter Wasser, Chem Ztg , 54: 118 (1930)

BERNHAUER, K Zum Chemismus der Citronensäurebildung durch Pilze I Die Säurebildung aus verschiedenen Kohlenstoffverbindungen II Die Citronensäurebildung aus Gluconsäure, Biochem Zeit , 197: 309–312 (1928)

———: "Die oxydative Gärungen," Verlag Julius Springer, Berlin, 1932.

———: "Gärungschemisches Praktikum," Verlag Julius Springer, Berlin, 1932

———: Biochemie der oxydativen Gärungen, Ergeb Enzymforsch , 3: 185–227 (1934).

——— und A IGLAUER. Über die Säurebildung aus Zucker durch *Aspergillus niger*. Biochem Zeit 286: 45 (1936)

———, und H KNOBLOCH Über die Säurebildung aus Zucker durch *A niger*, Biochem Zeit , 307: 298–306 (1911)

BIRKINSHAW, J H : Biochemistry of the Lower Fungi, Biol Rev , 12: 357 (1937)

BROWNE, C A : Citric Acid, Jour Ind Eng Chem , 13: 81 (1921)

BUTKEWITSCH, W S , et M S GAEWSKAYA Compt rend acad sci U R S S (N S), 3. 405 (1935)

———, E W MENZSCHINSKAJA, und E I TROFIMOVA. Zur Biochemischen Herkunft der Citronen- und Oxalsäure I Über die Bildung von Citronensäure aus Essigsäure, Biochem Zeit , 272: 290 (1934) II Die Mycelsubstanzen als ein Quelle der Säurebildung, Biochem Zeit , 272: 364 (1934)

CAHN, F J : Citric Acid Fermentation on Solid Materials, Ind Eng Chem , 27: 201 (1935)

CHALLENGER, F : The Production of Citric Acid by Fermentation Processes, Ind Chemist, 5: 181–184 (1929).

———, V SUBRAMANIAN, and T K WALKER· The Mechanism of the Formation of Citric and Oxalic Acids from Sugars by *Aspergillus niger*, I, Jour Chem Ind , January, 1927, pp 200–208.

CHATTERJEE, N P : Citric Acid from Molasses by *A niger*, Jour Indian Chem Soc , Ind & News Ed , 5: 201–205 (1942)

CHRZĄSZCZ, T , and J JANICKI. Recent Advances in the Fermentation Industries, Jour Soc Chem Ind , Nov 6, 1936

——— und K LEONHARD: The Production of Citric Acid from Lactic Acid and from Alcohol, Biochem Jour , 30. 1947–1952 (1936)

——— und L PEYROS· Optimale Bedingungen der Citronensäuren-häufung, sowie, einige Beobachtungen zur Theorie der Citronensäurebildung, Biochem Zeit , 280: 325 (1935).

——— und D TIUKOW: Biochemische Umbildungen der Essigsäure durch Schimmelpilze und über den Chemismus der Citronensäurebildung, Biochem Zeit , 229: 313 (1930)

——— und M ZAKOMORNY Biochemische Umbildung des Zuckers durch Schimmelpilze Die Umbildung de Fumarsäure, Anhäufung der Ameisensäure und der Chemismus der Oxalsäurebildung, Biochem Zeit , 259: 156 (1933)

—— und ——: Über den Mechanismus der Umbildung von Ameisensäure zu Oxal-und Kohlensäure durch verschiedene Schimmelpilze, *Biochem Zeit*, **279**: 64 (1935).

—— und ——: Über die Bedeutung der Äpfelsäure bei der Umbildung der Essigsäure in Citronensäure durch verschiedene Schimmelpilze, *Biochem Zeit*, **285**: 348–355 (1936).

Ciusa, R., e L Brülli: Sul meccanismo della fermentazione citrica -1, *Ann chim applicata*, **29**: 3–11 (1939).

Clutterbuck, P. W.: Recent Developments in the Biochemistry of Molds, *Jour Soc Chem Ind. (Trans. & Com.)*, **55**: 55T (1936).

Corbellini, A.: "L'utilizzazione dell'acido citrico alla sintesi dell'*l*-fenil 3-metil 5-pirazolone e derivati," Palermo, Ires, 1935

Cruess, W. V.: Getting the Most from Lemons. The Sicilian Citrus By-Products Industry is a Curious Mixture of Modern Technology and Ancient Tradition, *Chem. & Met. Eng*, **32**: 313–315 (1925).

Currie, J. N.: The Citric Acid Fermentation of *Aspergillus niger*, *Jour Biol Chem*, **31**: 15–37 (1917).

Das Gupta, G. C., K C. Saha, and B. C. Guha: The Fermentative Production of Citric and Oxalic Acids from "Gur" and Molasses, *Science and Culture*, **3** (No 7): 397–398 (1938).

——, and ——: The Fermentative Production of Citric and Oxalic Acids from Molasses, *Jour. Indian Chem. Soc., Ind. & News Ed.*, **3**: 64–74 (1940).

Doeloer, W. P., and S. C. Prescott: Citric Acid Fermentation, *Ind Eng Chem*, **26**: 1142 (1934).

Emde, H · Zur Theorie der Citronensäuregärung, *Biochem. Zeit.*, **275**: 373 (1935)

Fulmer, E I, and C H Werkman: "An Index to the Chemical Action of Microorganisms or the Non-nitrogenous Organic Compounds," Charles C Thomas, Publisher, Springfield, Ill, 1930

Galeano, S.: *Ann. merceol. siciliana*, **2**: 59 (1934).

Gerhardt, P., W. W Dorrell, and I. L Baldwin. Citric Acid Fermentation of Beet Molasses, *Jour Bact.*, **52** (No 5): 555–564 (1946).

Giordani, M : Fermentazione citrica, *Chimica e industria (Italy)*, **17**: 77–81 (1935)

Gudlet, M., V Kirsanowa, and V Makarowa: *Schriften Wiss Forsch Inst. Nahrungsmittelind. (U.S.S.R)*, **1**: 45 (1935)

Henneberg, W.: "Handbuch der Garungsbakteriologie," Vol. 1, Paul Parey, Berlin, 1926

Herrick, H T, and O. E. May: Fermentation as a Factor in Producing Organic Acids for Chemical Industry, *Chem. & Met. Eng*, **42**: 142–143 (1935)

Iwanoff, N N., and associates· *Proc. Inst. Sci. Research Food Ind. (U.S.S R)*, **3**: 3–153 (1935); **3**: 1–167 (1936).

—— and E S Zwetkoff: Biochemistry of Fungi, *Ann. Rev. Biochem*, **2**: 521 (1933); **5**: 585 (1936)

Karow, E O.. "The Production of Citric Acid in Submerged Culture," Doctor's Thesis, Rutgers University, New Brunswick, N.J, March, 1942

—— and S A Waksman: Production of Citric Acid in Submerged Culture, *Ind Eng Chem.*, **39**: 821–825 (1947).

Kostytschev, S., and V Berg: Conditions of Biochemical Production of Citric Acid, *Bull State Inst Appl. Microbiol. (U S S R)*, **5**: 8–27 (1933)

Lockwood, L B , and A. J Moyer: The Production of Chemicals by Filamentous Fungi, *Bot Rev*, **4**: 140–164 (1938).

May, O. E., and H. T. Herrick: Some Minor Industrial Fermentations, *Ind. Eng. Chem.*, **22**: 1172 (1930).

——— and ———: Production of Organic Acids from Carbohydrates by Fermentation, *U. S. Dept. Agr., Circ.* 216, May, 1932.

——— and ———: Some Practical and Theoretical Aspects of Mold Metabolism, *Jour. Bact.*, **28**: 145–151 (1934).

Molinari, E.: "Treatise on General and Industrial Chemistry," 2d ed., Part I, J. & A. Churchill, London, 1920.

Molliard, M.: *Compt. rend. soc. biol.*, **90**: 1395 (1924).

Palfi, T. Y., and M. A. Frantziuzova: Biochemical Citric Acid Production, *Proc. Inst. Sci. Research Food Ind. (U. S. S. R.)*, **3** (No. 4): 7–30 (1936).

Perlman, D.: Citric Acid Production by Various Strains of *Aspergillus niger*, Paper presented at Fourth International Congress of Microbiology.

———, W. W. Dorrell, and M. J. Johnson: Effect of Metallic Ions on the Production of Citric Acid by *Aspergillus niger*, *Arch. Biochem.*, **10** (No. 3): 131–143 (1946).

———, D. A. Kita, and W. H. Peterson: Production of Citric Acid from Cane Molasses, *Arch. Biochem.*, **11** (No. 1): 123–129 (1946).

———, H. A. Lardy, and M. J. Johnson: Determination of Citric Acid in Fermentation Media and Biological Materials, *Ind. Eng. Chem., Anal. Ed.*, **16**: 515–516 (1944).

Perquin, L. H. C.: "Bijdrage tot de Kennis der oxydatieve dissimilatie van *Aspergillus niger* van Tieghem," W. D. Meinema, Delft, 1938.

Pilhashy, B. M.: Citric Acid Obtained from Pineapples in California, *Chem. & Met. Eng.*, **33**: 559 (1926).

Porges, N.: *Am. Jour. Botany*, **19**: 559–567 (1932).

Photop'yakonov, O. P.: *Bull. State Inst. Agr. Microbiol. (U. S. S. R.)*, **5**: 231 (1933).

Quilico, A., e A. DiCapua: Sopra l'influenza del ferro sulla fermentazione citrica dell' *Aspergillus niger*, *Chimica e industria (Italy)*, **14**: 289 (1932).

Raistrick, H., and A. B. Clark: On the Mechanism of Oxalic Acid Formation by *Aspergillus niger*, *Biochem. J.*, **23**: 329 (1919).

Roberts, O., and D. Murphy: A Rapid Fermentation Method for the Production of Calcium Citrate and Calcium Gluconate from Beet Molasses, *Sci. Proc. Roy. Dublin Soc.*, **23**: 307–314 (1944).

Schwartz, W., and H. Lang: *Arch. Mikrobiol.*, **5**: 387 (1934).

Shu, P., and M. J. Johnson: Effect of the Composition of the Sporulation Medium on Citric Acid Production by *Aspergillus niger* in Submerged Culture, *Jour. Bact.*, **54** (No. 2): 161–167 (1947).

——— and ———: Citric Acid Production by Submerged Fermentation with *Aspergillus niger*, *Ind. Eng. Chem.*, **40** (No. 7): 1202–1205 (1948).

Taylor, J. N., and R. G. Boyd: United States Citric-acid Industry Now Self-sufficient, *Commerce Rpts.*, pp. 227–228, Jan. 26, 1931.

——— and R. A. Lord: World Trade in Citric Acid and Calcium Citrate, *Commerce Rpts.*, pp. 36–41, July 6, 1931.

Thom, C., and M. B. Church: "The Aspergilli," The Williams & Wilkins Company, Baltimore, 1926.

——— and J. N. Currie: *Aspergillus niger* Group Oxalic-Acid Production of Species of Aspergillus, *Jour. Agr. Research*, **7**: 1–15 (1916).

U. S. Tariff Commission Hearings, Tariff Act of 1922, Vol. I, Schedule I, pp. 1–9, 1929.

Von Loesecke, H. W.: A Review of Information on Mycological Citric Acid Production, *Chem. & Eng. News*, **23:** 1952–1959 (1945). (Contains 145 references.)

Walker, T. K.: Recent Developments in the Biochemistry of Bacteria, *Jour. Soc. Chem Ind. (Trans & Com.)*, **55:** 61T (1936).

———, V. Subramaniam, and F. Challenger: The Mechanism of the Formation of Citric and Oxalic Acids from Sugars by Aspergillus niger, II, *Jour. Chem. Ind*, pp. 3014–3054 (1927).

Warneford, F. H. S., and F. Hardy: Manufacture of Calcium Citrate and Citric Acid from Lime Juice, *Ind. Eng. Chem*, **17:** 1283 (1925).

Wehmer, C.: "Beiträge zur Kenntnis einheimischer Pilze," Hannover und Leipsig, Gustav Fischer, Jena, 1892

———: Note sur la fermentation citrique, *Bull. soc. chim*, **9:** 728–730 (1893)

———: Préparation d'acide citrique de synthèse, par la fermentation du glucose, *Compt rend*, **117:** 332 (1893).

———: Über zwei weitere freie Citronensäure bildende Pilze, *Chem. Ztg*, **21:** 1022–1023 (1897).

Wells, P. A , and H T. Herrick: Citric Acid Industry, *Ind. Eng. Chem*, **30:** 255–262 (1938).

———, A J. Moyer, and O. E. May: The Chemistry of the Citric Acid Fermentation. I The Carbon Balance, *Jour. Am. Chem Soc*, **58:** 555 (1936).

——— and G E. Ward: Fermentation Processes, *Ind. Eng Chem*, **31:** 172 (1939)

Wilson, C. P.: What Becomes of Second-grade Lemons? Citric Acid. The story of an Important Chemical Industry Developed in an Attempt to Salvage Fruit That Otherwise Would Be Wasted or Inefficiently Utilized, *Chem & Met. Eng*, **29:** 787 (1923).

Wünschendorff, M., et C Kiltan: Observations sur le métabolisme de l'Ustulina vulgaris L, *Compt. rend*, **187:** 572 (1928)

Some Patents

Bakonyi, L.: French Patent 819,173, Oct 12, 1937; also British Patent 474,897, Nov. 9, 1937; and Hungarian Patent 117,222, Nov 15, 1937

Barbisan, L E, and Soc d'Exploitations des Procédés L Cerini: French Patent 833,631, Oct 26, 1938

Bernhauer, K : German Patent 587819, Nov 30, 1933

Bleyer, D.: German Patent 434729, Oct 6, 1926

Boehringer, C F, und G M B H Soehne· British Patent 452,460, Aug 24, 1936, and German Patent 636135, Oct. 2, 1936

Cahn, F J : French Patents 675,236, and 675,237, Oct 29, 1929, also Canadian Patent 306,081, Nov 25, 1930, British Patent 345,368, March 26, 1931, British Patent 348,214, May 14, 1931, Production of Organic Acids by Fermentation, U S Patent 1,809,797, June 9, 1931; Citric Acid Manufacture, U S Patent 1,812,136, June 30, 1931, Citric Acid Manufacture, U S Patent 2,047,669, July 14, 1936

Chauncey Chemical Corporation· French Patent 819,279, Oct. 13, 1937; also British Patent 491,534, Sept 5, 1938

Distillers Company, Ltd : British Patent 287,604, Mar 27, 1928.

Eisenman, W , and M. D. Blumenfeld: Process for Production of Citric Acid by Fermentation, U S Patent 2,364,701, Dec 12, 1944

Falck, R : German Patent 426926, Mar 29, 1926 and 473727, Mar. 20, 1929

Fernbach, A., and J. L. Yuill: French Patents 610,121 and 610,122, May 29, 1926; also British Patents 266,114 and 266,415, Feb. 28, 1927, Process for the Production of Citric Acid, U.S. Patents 1,691,965 and 1,691,966, Nov. 20, 1928

Filosofov, M. S.: Russian Patents 17,505, June 30, 1927, and 19,622, Mar. 31, 1931.

Frey, A.: German Patent 567071, Mar. 30, 1933

Kanhäusen, F.: Process for the Production of Citric Acid by Fermentation, U.S. Patent 1,779,001, Oct. 21, 1930

Lilly, C. H.: Process of Mold Fermentation and Apparatus Therefor, U.S. Patent 1,936,983, Nov. 28, 1933

Lorentz, W. K.: German Patent 679847, Aug. 15, 1939, also British Patent 517,793, Feb. 8, 1040

Montan und Industrialwerke vorm Joh. Dav. Starck, French Patent 650,072, Jan. 4, 1929

Nenr, E.: German Patent 698905, Nov. 19, 1910

Nussbaum, J. French Patent 801,273, July 31, 1936

Peake, A. M. Production of Citric Acid, U.S. Patent 1,788,730, Jan. 13, 1931

Scholvien, K. German Patent 162622, Sept. 13, 1905

Shull, A. A. Russian Patents 30,272, July 21, 1933, and 35,187, Mar. 31, 1931

Szücs, J. Canadian Patent 251,180, June 30, 1925, also Austrian Patent 101,009, Sept. 25, 1925, German Patent 461356, June 13, 1928, Process for Producing Citric Acid by Means of Fermentation, U.S. Patent 1,679,186, July 31, 1928, Method of Producing Citric Acid by Fermentation, U.S. Patent 2,353,771, July 18, 1911

Waksman, S. A. Process of Fermentation, U.S. Patent 2,100,143, May 11, 1916

Wehmer, C. French Patent 228,551, Mar. 11, 1893, also British Patent 5,620, Dec. 9, 1893, Process of Making Citric Acid, U.S. Patent 515,033, Feb. 20, 1891; and German Patent 72937, Feb. 20, 1891

Woodward, J. C., R. S. Nicholls, and R. L. Snell: Canadian Patent 422,142, Aug. 15, 1911

Zahorski, B. Method of Producing Citric Acid, U.S. Patent 1,066,358, July 1, 1913

Zender, J. French Patent 803,630, Oct. 5, 1936, British Patent 462,350, Mar. 8, 1937, Process for Producing Citric Acid, U.S. Patent 2,072,919, Mar. 9, 1937; Production of Citric Acid, U.S. Patent 2,121,061, June 21, 1938.

CHAPTER XXVI

THE GLUCONIC ACID FERMENTATION

Gluconic acid ($C_6H_{12}O_7$) is produced by the oxidation of the aldehyde grouping of glucose. This conversion is represented by the following equation·

$$\underset{\text{Glucose}}{\overset{H_2}{\underset{\overset{|}{OH}}{\overset{|}{C}}}-\left[\overset{H}{\underset{\overset{|}{OH}}{\overset{|}{C}}}\right]_4-\overset{O}{\underset{H}{C}}} +\tfrac{1}{2}O_2 \quad \underset{\text{Gluconic acid}}{\overset{H_2}{\underset{\overset{|}{OH}}{\overset{|}{C}}}-\left[\overset{H}{\underset{\overset{|}{OH}}{\overset{|}{C}}}\right]_4-\overset{O}{\underset{OH}{C}}}$$
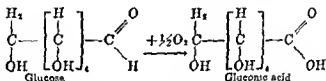
Methods of Production.—By chemical means, gluconic acid may be prepared from glucose by oxidation with a hypochlorite solution. Gluconic acid may be produced, in a second method, by the electrolysis of a solution of the sugar containing a measured amount of a bromide.

Gluconic acid may be produced by the fermentation of nutrient solutions of glucose by many molds and bacteria. Great differences exist, however, between the various microorganisms in respect to their adaptability for commercial use. Some, of course, produce only small yields of gluconic acid; some may be classed as slow fermenters; some lack stable cultural characteristics

Historical.—The production of this acid by microorganisms was first observed by Boutroux in 1878. *Mycoderma aceti* (*Acetobacter aceti*) produced a substance from glucose which was first thought to be lactic acid but which was identified two years later as gluconic acid.

Molliard, in 1922, discovered gluconic acid as a product of mold fermentation, along with citric and oxalic acids. The acid was produced in sucrose mashes as a result of the action of *Sterigmatocystis nigra* (*Aspergillus niger*). Molliard later developed some optimum conditions for the fermentation.

In 1924 Bernhauer discovered a strain of *A. niger* that would produce gluconic acid almost exclusively when in the presence of *calcium carbonate*. Bernhauer has shown that thin mats grown at a low temperature with a low supply of nitrogen favored the production of gluconic acid. On the other hand, higher yields of citric acid were obtained when the fermentation temperature was relatively high, the nitrogen supply high, and the mats heavy

Preparation by Mold Fermentation.—An extensive study of the gluconic acid fermentation has been carried out over a period of years by May, Herrick, Moyer, Hellbach, Wells, Stubbs, and others of the U.S. Department of Agriculture In 1938 it was shown by Gastrock, Porges, Wells, and Moyer that gluconic acid could be successfully produced on a pilot-plant scale from refined corn sugar (commercial glucose) In general, gluconic acid may be produced by mold fermentation, using the shallow-pan method or using submerged mold growths under increased air pressure. The latter method is much superior to the former, not only in respect to the time required for the fermentation but also in the yield and in the ease of production.

The Shallow-pan Method.—In the shallow-pan method, a sterile nutrient glucose solution is inoculated with the mold and incubated in cabinets designed to prevent contamination The mycelial mat develops as in the citric acid fermentation, and glucose is transformed to gluconic acid by the mold enzyme—an oxidase. During incubation the mat must not be disturbed

Herrick and May, using *Penicillium purpurogenum* var *rubrisclerotium* (Thom No 2,670), found that gluconic acid could be produced to the exclusion of other acids. After considerable experimentation, they adopted the following nutrient salt solution.

	Grams per Liter of Glucose Solution
$MgSO_4\,7H_2O$	0 25
KCl	0 05
$Na_2HPO_4\,12H_2O$, or H_3PO_4	0 1
$NaNO_3$	1 0

A 20 to 25 per cent concentration of glucose was most conducive to high yields of the acid, while a temperature of $25°C$ was most favorable. Lower temperatures resulted in smaller yields and required a longer time for the completion of the fermentation At higher temperatures, the mycelial mats formed rapidly, but they sank, with resultant low yields. The ratio of surface area to volume was important—one of 0.25 to 0 30 was found to be best for practical purposes As the ratio of square centimeters to cubic centimeters approached unity, 82 per cent of the theoretical yield of gluconic acid was produced, but unity is an impractical ratio to employ. With a ratio of **0.16**, only 30 per cent of the theoretical yield could be obtained.

Agitation of the glucose solution was considered. When the concentration of sugar was low, agitation was advantageous. The effect of agitation decreased with the increase of sugar concentration There was

no advantage to be gained from agitating a 20 to 25 per cent solution of glucose.

A pH range of 3 to 6.4 was satisfactory for the fermentation. Altering the pH values from this range brought about no advantages.

The rate of oxidation of glucose was greatest between the fifth and ninth days. When the culture liquor in a fermentation was replaced with a fresh, sterile glucose solution, an active oxidation commenced at once.

Under the foregoing conditions yields of 55 to 65 per cent of the theoretical were produced.

Moyer, May, and Herrick[1] discovered a mold (secured from the collection of Dr. Thom) which possessed biochemical and vegetative vigor, qualities lacking in the culture of *Penicillium luteum purpurogenum* investigated earlier, and which produced good yields of gluconic acid. Out of the more than 50 *Penicillium* species investigated, this mold, *P. chrysogenum*, culture 5,034.11, showed the greatest capacity for producing gluconic acid. The following medium was used for the production of gluconic acid under nearly optimum conditions: 20 to 25 per cent commercial glucose, and 3.00 g. $NaNO_3$, 0.300 g. KH_2PO_4, and 0.250 g. $MgSO_4 \cdot 7H_2O$ per liter. A surface area to volume ratio of 0.4 to 0.5 was used. Under the foregoing conditions, 60 per cent of the glucose was oxidized to gluconic acid in 8 to 10 days at 30°C. Ferric chloride acted as a stimulant to growth and acid production when nutrients of high purity were used.

Selection of Pan.—In the production of gluconic acid through fermentation by molds, using the shallow-pan method as described above, a very important consideration is that of the selection of an appropriate material for the construction of the pan. Several requirements must be satisfied, namely, noncorrosiveness to acid, lack of toxicity, low cost, and durability. Iron, zinc, and ordinary aluminum are attacked by acid. Nickel, lead, copper, and monel metal are somewhat toxic. Glass would be satisfactory but it is easily broken and expensive. Block tin and bakelite may be used, but they are also expensive. Iron, when enameled, is satisfactory, but it is easily chipped, unwieldy, and expensive. Lacquers are not good. Aluminum of a high purity, 99.45 per cent aluminum, and containing less than 0.1 per cent of copper and manganese has given good results.

Aeration, Submerged Growths.—Schreyer in 1928 demonstrated that agitation, aeration, and the use of calcium carbonate ($CaCO_3$) increased the yield of gluconic acid from four to six times that of cultures which

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[1] MOYER, A. J., O. E. MAY, and H. T. HERRICK, *Centr. Bakt. Parasitenk.*, Abt II, **95:** 311 (1936).

were not aerated. *Aspergillus fumaricus* was used in glucose solutions. The yield of citric acid by this organism was not altered by the change in conditions. Thies (1930) used the same mold but bubbled oxygen instead of air through the medium and obtained similar results. Currie, Kane, and Finlay (1933) reported yields of gluconic acid as high as 90 per cent when the mold growth was submerged, when the medium was maintained in a high degree of agitation by means of a stirring device, and when air was drawn in large quantities into the solution.

Laboratory Scale.—In work carried out by the Color and Farm Waste Division of the U S Department of Agriculture,[1] gluconic acid was produced in high yields by submerged growths of *Penicillium chrysogenum* under increased air pressure The medium used contained 3 g. of $NaNO_3$, 0.15 g of KH_2PO_4 and 0 125 g $MgSO_4 \cdot 7H_2O$ and the equivalent of 20 per cent of pure glucose per liter. The addition of 1 g of calcium carbonate ($CaCO_3$) for each 4 g of glucose caused an increase in the yields of acid and likewise served as a supporting medium.

The fermentation was carried out in 500-cc. gas-washing bottles with sintered-glass false bottoms. In each bottle, 200 cc. of nutrient medium were sterilized (at 15 lb. pressure for 15 min.), cooled, and inoculated with mold spores Calcium carbonate, when used, was sterilized separately and added aseptically Filtered, humidified air was permitted to enter through the sintered-glass bottoms of the bottles at a controlled rate (40 cc. per min), thus providing agitation and aeration. Under the foregoing conditions, 80 to 87 per cent yields of gluconic acid were obtained at a temperature of 30°C. when the air pressure was maintained at 3 atmospheres or above (in an autoclave) and calcium carbonate was used in the ratio indicated.

The Rotary Drum (Laboratory Size).—In 1935, Herrick, Hellbach, and May[2] described the rotary drum, an apparatus for producing gluconic acid by submerged mold growths The drum was developed in an attempt to find the most suitable type of equipment for the industrial production of gluconic acid by molds. It was constructed of aluminum containing less than 0.1 per cent of manganese and copper Buckets and baffles were attached to the inside shell of the drum, which served to bring the oxygen of the air into intimate contact with the nutrient glucose solution and the mold. Facilities were available for sterilizing the drum with steam before charging it with the medium, calcium carbonate ($CaCO_3$), and inoculum.

During operation, sterilized air is introduced through an inlet and

[1] May, O E , H T Herrick, A J Moyer, and P A Wells, *Ind Eng Chem*, 26: 575 (1934)

[2] Herrick, H T , R Hellbach, and O E. May, *Ind Eng Chem*, 27: 681 (1935).

allowed to accumulate until the desired pressure is built up in the drum.
Thereafter a definite quantity of air flows into and out of the drum at a
constant rate. The drum is rotated by means of belts applied to its
outside flanges.

The rotary drum is shown in the accompanying photograph (Fig. 81).
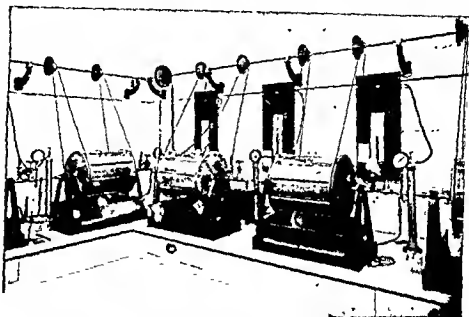
Fig. 81—Laboratory-scale fermentation apparatus. [*Courtesy of Gastrock, Porges, Wells, and Moyer, Ind Eng Chem , 30 · 782 (1938) *]

Initial experimentation with the rotary drum indicated that the time
required for the fermentation was considerably reduced by its use. The
following table compares the production of gluconic acid by different
methods.

TABLE 127 —THE PRODUCTION OF GLUCONIC ACID BY DIFFERENT METHODS[1]

Type of fermentation	Organism	Fermentation vessel	Yield of acid (theo-retical),[2] per cent	Fermenta-tion period, days
Surface	*Penicillium luteum purpurogenum*	Shallow pan (alumi-num)	57 4	11
Submerged (pres-sure).	*P. chrysogenum*	Glass bottle (sintered glass, false bottom)	80 4	8
Submerged (pres-sure).	*P. chrysogenum*	Rotary drum (alumi-num)	80 0	2.2

[1] WELLS, P. A., A J. MOYER, J J STUBBS, H T. HERRICK, and O. E. MAY, *Ind Eng Chem , 29: 653 (1937)*.

[2] From 20 per cent glucose solutions.

Details of the Rotary-drum Process (Laboratory Scale) —In the rotary-drum process, the selection of an appropriate organism, the maintenance of an optimum oxygen supply, the composition of the various media employed, and the use of calcium carbonate in the process are important.

THE ORGANISM.—*Penicillium chrysogenum*, an organism used in producing gluconic acid by submerged growths under pressure, does not readily produce the large quantities of spores required for inoculating mashes. *Aspergillus niger* (strain No. 67 of the Industrial Farm Products Research Division) was selected by Wells, Moyer, and their associates because it possessed certain desirable characteristics—it readily produced spores and uniform fermentations.

THE OXYGEN SUPPLY.—The effectiveness of the oxygen supply in the rotary-drum process is controlled by three factors, agitation, air flow, and air pressure. Upon the efficiency of these factors depends, largely, the success of the fermentation, provided the medium and inoculum are satisfactory

Optimum results were obtained when the rotation of the drum (see photograph on page 610) was 13 r p m. Higher speeds produced excessive frothing of the medium Air flows of 400 to 1,200 cc per min for a volume of 3,200 cc of mash were satisfactory. (Costs increase as the amount of air used increases) By maintaining the gauge at pressures of 30 to 45 lb per sq in., excellent results were obtained. A pressure of 30 lb per sq. in was considered most practical, however, since higher pressures increased the danger of damaging the equipment and producing leaks. The following table illustrates the effect of air pressure on the fermentation of glucose to gluconic acid by *Aspergillus niger:*

TABLE 128 —THE EFFECT OF VARIOUS AIR PRESSURES ON GLUCONIC ACID YIELDS[1]

(Air flow, 1,200 cc per min , speed, 13 r p m , volume of medium, 3,200 cc , glucose available, 495 g , fermentation period, 18 hr , temperature. 30°C.)

Gauge pressure, lb per sq in (kg per sq cm)	Glucose consumed, grams	Gluconic acid produced, grams	Gluconic acid yield based on	
			Glucose consumed, per cent	Glucose available, per cent
5 (0 35)	178	173	89 1	32 1
15 (1 05)	257	258	92 1	47 9
30 (2 11)	336	351	93 0	65 1
45 (3 16)	429	454	97 1	84 2

[1] WELLS, P. A , A J. MOYER, J J STUBBS, H T HERRICK, and O E MAY, Ind. Eng Chem , 29 653 (1937)

MEDIA.—The effect of variations in the nature and quantities of the nutrient salts and other substances and in the amount of glucose on the results obtained with a given organism is well illustrated in the gluconic acid fermentation.   A medium of one composition is used for maintaining the growth of the culture, a second medium for inducing sporulation, a third medium for stimulating germination, and a fourth medium for the production of gluconic acid.   Inasmuch as the media used for the production of gluconic acid on a pilot-plant scale vary but slightly from those used for the preparation of the acid on a laboratory scale,[1] a summary of the former will be given (courtesy of the authors and editors).

TABLE 129.—SUMMARY OF MEDIA USED FOR GLUCONIC ACID PRODUCTION BY *Aspergillus niger*[1]

Ingredient	A, culture	B, sporulation	C, germination	D, fermentation
Grams per liter:				
Refined corn sugar[2]	30 0	50.0	100 0	Varies
MgSO₄·7H₂O	0 10	0 12	0 25	0.156
KH₂PO₄	0 12	0 144	0 30	0 188
(NH₄)₂HPO₄	None	0 56	0 30	0 388
NH₄NO₃	0 225	None	None	None
Peptone	0 25	0 20	0 02	None
Potatoes	200	None	None	None
Agar	20.0	1 5	None	None
CaCO₃	4 0	None	37 5*	20 0*
Beer, cc per liter	None	45	40	None
Kind of water	Distilled	Distilled	Tap	Tap

[1] GASTROCK, E A., N PORGES, P A. WELLS, and A J MOYER, *Ind. Eng. Chem.*, 30: 782 (1938).
[2] Refined corn sugar, containing 91 5 per cent of dextrose and corresponding closely to dextrose monohydrate, was used in almost all the research
* Separately sterilized.

Technique of Preparing Inoculum.—*Aspergillus niger*, strain 67, is cultured for 7 days at 30°C on slants of medium A.   These slant cultures are used to inoculate at least twenty 1-liter Erlenmeyer flasks, each containing 150 cc. of medium B.   The flasks are incubated at 30°C. for 7 . . . . transferred to a
. . . . ughly broken up by means of a mechanical agitator.   The contents of the flask are divided into two portions, and each is placed in a small rotary drum—the total charge in each drum occupying approximately one-third of its total volume.   The following conditions are maintained during germination:

[1] MOYER, A. J., P. A. WELLS, J. J. STUBBS, H. T. HERRICK, and O. E. MAY, *Ind. Eng. Chem.*, 29: 777 (1937)

1 Tube culture
2
3
4
5
6
7 Handhole, commercial dextrose, nutrients, and sterile calcium carbonate are added here
8 Bag filter
9 Aluminum tank for neutralization and crystallization, calcium hydroxide milk is added here,
10 Centrifuge (stainless-steel basket, aluminum-lined curb)
11 Vacuum evaporator for mother liquors
12 Condenser
13 Vacuum drier
14 To calcium gluconate storage

Fig 82 —Flow sheet of gluconic acid production. [*Courtesy of Gastrock, Porges, Wells, and Moyer, Ind Eng Chem , 30 . 782 (1938)*]

Air pressure.	**30** lb. per sq. in. gauge (155 mm. Hg).
Air flow ...	**375** cc. per liter per min.
Speed of rotation	5.8 r.p.m.
Temperature	30°C.
Time. .	24 hr.

By permitting the spores to germinate in medium *C*, the fermentation period is shortened and less inoculum is required for a given volume of fermentation medium *D*.

The Pilot-plant Fermenter.—The solutions containing the germinated spores are transferred aseptically to a large-scale fermenter containing medium *D*. Wells, Lynch, Herrick, and May[1] have described this
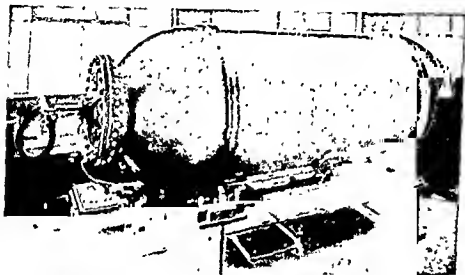
Fig. 84.—Large-scale fermentation apparatus. (*Courtesy of Gastrock, Porges, Wells, and Moyer, Ind. Eng. Chem., 30: 782 (1938).*)

fermenter. The drum is 3 by 6 ft. and is constructed of aluminum sheet, containing 99.5 per cent aluminum and less than 0 1 per cent of copper, iron, and manganese. End castings are made of an aluminum-silicon alloy. The tank holds 420 gal., but ordinarily about 140 gal. of charge are added—larger charges increase the fermentation time. Buckets and baffles are welded to the interior of the shell and function as in the small rotary fermenters. Facilities are provided for sterilizing the drum and its contents. An aluminum pipe, which is attached to the drum's interior surface and looped back and forth around the ends of the buckets and baffles, extends around one-third of the periphery of the drum on the portion opposite the hand hole By connecting one end of the tubing to a steam line and the other to a drain, steam may be supplied for sterilization purposes (Fig. 84). Means are available for filling and emptying the

[1] Wells, P A, D F J. Lynch, H T Herrick, and O. E May, *Chem & Met Eng*, 44: 188 (1937)
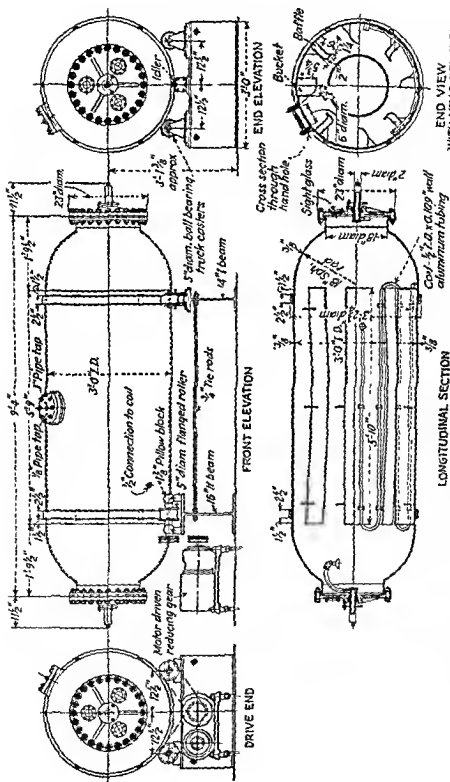
FIG. 84.—Rotary drum fermenter [Courtesy of Gastrock, Porges, Wells, and Moyer, Ind. Eng. Chem., 30: 782 (1938).]

drum. Sterile, humidified air is passed through the drum under pressure. For further details, the reader is referred to the papers cited in the foregoing paragraphs.

Neutralization of Gluconic Acid.—High acidity inhibits the fermentation. The rate at which glucose is converted to gluconic acid is more rapid in the presence of undissolved calcium carbonate than in the presence of free acid. It has been shown by Gastrock and his associates
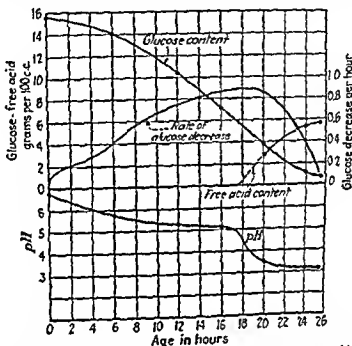
Fig. 85 —Correlation of pH with utilization of glucose (15 6 g per 100 ml.) by *Aspergillus niger* at 6 0 r p m *[Courtesy of Gastrock, Porges, Wells, and Moyer, Ind Eng. Chem., 30: 782 (1938)]*

that the use of 26 g. of calcium carbonate per liter of fermentation medium satisfactorily controls the pH of the medium. This quantity of calcium carbonate dissolves readily in the medium and is equivalent to 102.1 g. of gluconic acid per liter. The use of an excess of calcium carbonate retards the fermentation, since the calcium gluconate formed tends to crystallize out and prevent free contact of the medium with the mold.

The greatest activity in the medium coincides quite closely with the point at which free acid develops. Until the free acid develops, the pH of the medium remains close to 5.5; it then drops rapidly to a value of 3.5 or less. Free acid, with the drop in pH, usually appears about 17 hr after the start of the fermentation. During the drop in pH, the activity becomes diminished. Thus a pH of 5.5 is favorable for the production of the acid, since the maximum rate of conversion is at pH values of

greater than 5. The foregoing figure correlates pH with glucose utilization.

Semicontinuous Production of Gluconic Acid.—A process has been developed by Porges and his associates[1] in which gluconic acid may be produced successfully on a semicontinuous basis and which possesses several advantages over the single-batch method previously used.

The oxidations were carried out in laboratory- and semiplant-size fermenters, which have already been described, under the following conditions: an air pressure of 30 lb. per sq. in. (gauge), an air flow of 375 cc per liter of medium per minute, a rotation speed of 9.5 r.p.m. and a temperature of 30°C

As a result of research, a glucose concentration of approximately 11.5 g per 100 cc. was found to be optimum, rather than one of 15 g per 100 cc. The use of lower glucose concentrations resulted in shorter fermentation periods, but in lowered efficiencies of production

The initial charge of nutrient medium in a fermenter was inoculated with a pregerminated culture. The inoculum for each subsequent charge was secured by floating the mycelia from the previously completely fermented medium. A fermentation was judged to be complete when less than 1 per cent of glucose remained unconverted or when the rate of acid production was negligible in comparison to that of active fermentation. Flotation was accomplished by reducing the pressure in the fermenter to that of the atmosphere for a period of 35 min. During this interval most of the mycelia rose to the upper portions of the medium Subsequently the lower 80 per cent of the charge was removed, and a new charge of medium was introduced. The upper 20 per cent of the charge retained from the previous fermentation contained most of the active mycelia—more than 85 per cent

Under the foregoing conditions, a fermentation (excluding the initial one) was usually complete in a little more than 9 hr

As many as 13 successive fermentations have been carried out, using this general procedure, with no apparent loss in efficiency, according to Porges and his associates.

Later Porges, Clark, and Aronovsky (1941) showed that the mycelia of *A. niger* 67 could be recovered by pressure filtration and re-used in nine successive fermentations of media containing 16 g of glucose and about 2.6 g. of calcium carbonate per 100 ml. The pressure filter was constructed of aluminum since this metal was nontoxic to *A niger* and noninhibitory to the fermentation. Previous studies had shown that the use of an iron filter was unsatisfactory because of the inhibitory effect

excess of calcium carbonate, and boric acid (1,500 p.p.m. boron) is illustrated in Fig. 87.

Fig. 87.—Gluconic acid production from glucose by repeated reuse of *A. niger*, 3 mycelium. [*Courtesy of A. J. Moyer, E. J. Umberger, and J. J. Stubbs, Ind Eng. Chem., 32: 1379 (1910).*]

Industrial Production.—The production of gluconic acid on an industrial basis from glucose solutions containing nutrient salts and calcium carbonate and using *A. niger* has also been described by Williams (1945).

Additional Factors Affecting the Final Yield.—Glucose, mannose, mannite, and maltose may be used as sugars in the production of gluconic acid. Sucrose, lactose, and fructose are not suitable sugars for the fermentation for obvious reasons.

Toluol, potassium cyanide, and carbon monoxide inhibit the formation of gluconic acid when oxygen is available.

Uses of Gluconic Acid.—Gluconic acid is used principally as a pharmaceutical. Calcium gluconate is preferred to calcium lactate as a means of supplying calcium to children and to pregnant mothers. The injection of calcium gluconate into cows suffering with milk fever has produced excellent cures.

References on Gluconic Acid Fermentation

AMELUNG, H : Beitrage zur Säurebildung durch *Aspergillus niger*, *Zeit physiol. Chem.*, 166: 161 (1927).

Angeletti, A.: Azione di alcuni funghi su soluzioni di aldosi   Nota III. Formazione di acido d-gluconico per mezzo del *Penicillium crustaceum* (L) Fries, *Ann chim applicata*, **22**: 59 (1932).

———— e F F Cerruti. Sull'azione di alcuni funghi su soluzioni di aldosi, *Ann. chim. applicata* **20**: 424 (1930)

———— e L Merlo *Ann chim applicata*, **24**: 468 (1934).

Bernhauer, K.: Zum Problem der Säurebildung durch *Aspergillus niger* (Vorläufige Mitteilung), *Biochem Zeit*, **153**: 517 (1924).

————: Über die Säurebildung durch *Aspergillus niger*. I Allgemeines und Methodisches bei der Untersuchung der Säurebildungsvorgänge, *Biochem. Zeit*, **172**: 296 (1926)

————: Über die Charakterisierung der Stamme von *Aspergillus niger* auf Grund ihres biochemischen Verhaltens I Mitteilung. Vergleichende Untersuchungen über die Säurebildung durch verschiedene Pilzstämme, *Biochem Zeit.*, **197**: 278 (1928).

————: Über die Säurebildung durch *Aspergillus niger* IV Mitteilung. Die Bedeutung der Mycelentwicklung für die Säurebildung, *Biochem. Zeit*, **197**: 287 (1928)

————, "Die Oxydativen Gärungen," Verlag Julius Springer, Berlin, 1932.

————, "Gärungschemisches Praktikum," 2d ed, Verlag Julius Springer, Berlin, 1939

————und B Görlich Oxydationen mittels Essigbakterien. IV. Über die Bildung von 2-Keto-gluconsäure durch *Bact gluconicum*, *Biochem. Zeit*, **280**: 367 (1935).

———— und K Iragang Oxydationen mittels Essigbakterien III. Über die Bildung einer reduzierenden Zuckercarbonsäure ("Aldehydgluconsäure") neben 5-Ketogluconsäure, *Biochem Zeit*, **280**: 360 (1935).

———— und K. Schön: Oxydationen mittels *Bacterium xylinum* II Über die Bildung von Glucon- und 5-Ketogluconsäure, *Zeit physiol Chem*, **180**: 232 (1929)

Birkinshaw, J H. Biochemistry of the Lower Fungi, *Biol Rev*, **12**: 357 (1937)

———— and H Raistrick. Studies in the Biochemistry of Micro-organisms XVII The Products of Glucose Metabolism formed by Various Species of Fungi, *Trans Roy Soc London, Ser B*, **220**: 331 (1931)

Boutroux, L Sur une fermentation nouvelle du glucose, *Compt rend*, **91**: 236 (1880)

———— Sur l'acide gluconique, *Compt rend*, **104**: 369 (1887)

Brown, A J · The Chemical Action of Pure Cultivations of *Bacterium aceti*, *Jour. Chem Soc*, **49**: 172 (1886)

Butkewitsch, W Über die Bildung der Glucon- und Citronensäure in den Pilzkulturen auf Zucker, *Biochem Zeit*, **154**: 177 (1924)

———— Über die Säurebildung bei den Pilzen, *Biochem Zeit*, **182**: 99 (1927)

Butlin, K R Survey of the Biochemical Activities of the Acetic Acid Bacteria, Chemical Research, Special Report 2, H M Stationery Office, London, 1936

Chrzaszcz, T, und D Tiukow· Über die Säurebildung der Penicilliumarten (Link), *Biochem Zeit*, **204**: 106 (1929)

———— und ———— Die Stärkebildung bei den Schimmelpilzen (*Penicillium* Link), wie auch ihr Zusammenhang mit der Säurebildung, *Biochem Zeit*, **207**: 39 (1929)

———— und ———— Der Zusammenhang der Stärkebildung mit der Säurenanhäufung bei den Schimmelpilzen (*Penicillium*), *Biochem Zei t*, **222**: 243 (1930).

Falck, R, und S N, Kapur Über Gluconsäure—Bildung durch Fadenpilze, *Ber*, **57B**· 920 (1924)

Frost, C, J L St. John, and H W Gerritz Calcium Gluconate from Juice of Cull and Surplus Apples, *Ind Eng. Chem*, **28**: 75 (1936).

GASTROCK, E. A., and N. PORGES: Gluconic Acid Production on Pilot-plant Scale —Effect of Variables on Production by Submerged Mold Growths, *Ind Eng. Chem.*, 30: 782 (1938).

HAEHN, M.: *Deut. Essigind*, 33: 385 (1929).

HENNEBERG, W.: *Deut. Essigind.*, 2: 145 (1898).

HERMANN, S: *Bacterium gluconicum, ein in der sogenannten Kombucha (japanischer oder indischer Teepilz) vorkommender Spaltpilz, Biochem. Zeit.*, 205: 297 (1929)

———: *Über die Bildung von Gluconsäure und Ketogluconsäure durch Bacterium gluconicum, Bacterium xylinum und Bacterium xylinoides, Biochem Zeit,* 214: 357 (1929).

——— und P. NEUSCHUL: *Zur Biochemie der Essigbakterien zugleich ein Vorschlag für eine neue Systematik, Biochem Zeit*, 233: 129 (1931).

——— und P. NEUSCHUL *Centr. Parasitenk.*, Abt. II, 93: 25 (1935).

HERRICK, H T., R. HELLBACH, and O E MAY: Apparatus for the Appl . . .
. . . .
. . . . gogenum Group. II. Some Optimal Conditions for Acid Formation, *Jour. Biol. Chem.*, 77: 185 (1928)

HERZFELD, und Z. LENART: *Vereinfachtes Verfahren zur Darstellung von Glucon-säure, Ver. deut. Zucker-Ind.*, 69: 122 (1919).

KARDO-SYSSOJEVA, E : *Über die Bildung von Gluconsäure durch Aspergillus niger, Biochem. Zeit.*, 266: 337 (1933)

KOSTYTSCHEW, S., and W TSCHESNOKOV: *Compt. rend. acad. sci. U.R.S.S.,* 13: 193 (1927).

LOCKWOOD, L B., and A J MOYER: The Production of Chemicals by Filamentous Fungi, *Bot. Rev*, 4: 140 (1938)

MAY, O. E, and H T HERRICK The Production of Organic Acids by Fermentation, *U.S. Dept. Agr.*, Circ. 216, May, 1932

——— and ———, Some Practical and Theoretical Aspects of Mold Metabolism, *Jour. Bact.*, 28: 145 (1934).

———, ———, A. J MOYER, and R. HELLBACH: Semi-plant Production of Gluconic Acid by Mold Fermentation, *Ind. Eng Chem.*, 21: 1198 (1929).

———, ———, ———, and P. A. WELLS: Gluconic Acid. Production by Submerged Mold Growths under Increased Air Pressure. *Ind. Eng. Chem ,* 26: 575 (1934).

———, ———, C. THOM, and M. B. CHURCH: The Production of Gluconic Acid by the *Penicillium luteum-purpurogenum* Group, I, *Jour. Biol Chem ,* 75: 417 (1927).

MOLLIARD, M · Sur une nouvelle fermentation acide produite par le *Sterigmatocystis nigra, Compt. rend ,* 174: 881 (1922).

———: Nouvelles recherches sur la formation d'acides organiques par le *Sterigmato-cystis nigra en milieux déséquilibres, Compt rend.,* 178: 41 (1924).

MOYER, A. J., O. E. MAY, and H. T. HERRICK: Production of Gluconic Acid by *Penicillium chrysogenum, Centr. Bakt. Parasitenk ,* Abt. II. 95: 311 (1936).

———, E. J. UMBERGER, and J. J. STUBBS: Fermentation of Concentrated Solutions of Glucose to Gluconic Acid, *Ind. Eng Chem ,* 32: 1379-1383 (1940).

———, P. A. WELLS, J J. STUBBS, H. T. HERRICK, and O. E. MAY: Gluconic Acid Production. Development of Inoculum and Composition of Fermentation Solution for Gluconic Acid Production by Submerged Mold Growths under Increased Air Pressure, *Ind. Eng. Chem.*, 29: 777 (1937).

MÜLLER, D : Studien über ein neues Enzym Glykoseoxydase, I, *Biochem. Zeit ,* 199: 136 (1928).

————: Studien über ein neues Enzym Glykosooxydase, II, Biochem. Zeit., 205: 111 (1929)

PONCES, N, T F CLARK, and S I ARONOVSKY: Gluconic Acid Production—Repeated Recovery and Re-use of Submerged Aspergillus niger by Filtration, Ind. Eng Chem., 33: 1065–1067 (1941).

————, and E A GASTROCK: Gluconic Acid Production. Repeated Use of Submerged Aspergillus niger for Semicontinuous Production, Ind. Eng. Chem., 32: 107–111 (1940)

SCHREYER, R   Vergleichende Untersuchungen über die Bildung von Gluconsäure durch Schimmelpilze, Biochem. Zeit., 249: 295 (1931).

SUZUKI, Y   Bull Agr. Chem Soc (Japan), 5: 10 (1929)

TAKAHASHI, T, and T ASAI: On Gluconic Acid Fermentation.   I. On Bacterium Hoshigaki var rosea nov spec, Centr Bakt Parasitenk, Abt II, 82: 390 (1930).

———— and ————: ———— Jour Agr Chem Soc (Japan), 6: 17, 23 (1930)

————: On Gluconic Acid Fermentation   III. On Bacterium Hoshigaki var glucuronicum I, II & III, nov spec, Centr. Bakt Parasitenk, Abt. II, 87: 385 (1933)

TAMIYA, H, und T IIDA. Vergleichende Studien über die Säurebildung, die Atmung, die Oxydasereaktion und das Dehydrierungsvermögen von Aspergillusarten, Acta phytochim (Japan), 4: 313 (1928–1929).

TANAKA, K Zur Physiologie der Essigbakterien, Acta phytochim (Japan), 7: 205 (1933)

VISSER'T HOOFT, F: Biochemische Onderzoekingen over het geslacht Acetobacter, W D Meinema, Delft, 1925

WEHMER, C   Abnahme des Saeuerungsvermögens und Änderung der Säure bei einem Pilz (Gluconsäure-statt Fumarsäure-Garung), Biochem. Zeit., 197:418 (1938)

WELLS, P A, D F J LYNCH, H T HERRICK, and O. E. MAY: Translating Mold Fermentation Research to Pilot Plant Operation, Chem & Met Eng 44: 188 (1937)

————, A J MOYER, J J STUBBS, H. T. HERRICK, and O E MAY: Gluconic Acid Production   Effect of Pressure, Air Flow, and Agitation on Gluconic Acid Production by Submerged Mold Growth, Ind. Eng Chem., 29: 653 (1937).

WILLIAMS, A E : Production of Gluconic Acid, Mfg Chemist, 16: 239–241 (1945).

Some Patents

BERNHAUER, K · French Patent 707,614, 1931

———— and L SCHULHOF Production of Gluconic Acid, U S Patent 1,849,053, Mar. 15, 1932

CURRIE, J N, and R H CARTER Production of Gluconic Acid, U S Patent 1,896,811, Feb 7, 1933

————, J H KANE, and A FINLAY. Process for Producing Gluconic Acid by Fungi, U S Patent 1,893,819, Jan 10, 1933, also British Patent 370,609, 1930

FALCK, R German Patent 553238, 1924

————. German Patent 556761, 1931

HERRMANN, S German Patent 522147, 1931.

————. Austrian Patent 127,373, 1932

————. Austrian Patent, 133,139, 1933

HERRICK, H. T., and O. E. MAY: Process for the Manufacture of Gluconic Acid, U.S. Patent 1,726,067, Aug. 27, 1929.

PASTERNACK, R., and W. R. GILES: Process for the Preparation of Gluconic Acid and Its Lactones, U.S. Patent 1,942,660, Jan. 9, 1934.

STOLL, A.: Process for the Oxidation of Aldoses, U S Patent 1,648,368, Nov. 8, 1927.

——— and W. KUSSMAUL: Oxidation of Aldoses, U S Patent 1,703,755, Feb 26, 1929.

TAKAHASHI, T.: Process for Preparing Gluconic Acid, U.S Patent 1,953,694, Apr. 3, 1934.

THE FUMARIC ACID FERMENTATION

Fumaric acid (HOOC·CH=CH·COOH), an unsaturated acid, is produced by several molds, principally species of the genus *Rhizopus* Other genera of the family *Mucoraceae*, namely, *Circinella*, *Cunninghamella*, and *Mucor*,[1] also have the ability to produce fumaric acid from nutrient sugar solutions At least one species of *Aspergillus* and one of *Penicillium* have been credited with the production of this acid.

Ehrlich[2] (1911) first reported on the formation of fumaric acid by molds

There is much variation in acid production even in strains that appear to be identically morphologically For example, Foster and Waksman[3] have reported the case of acid production by races of a strain of *Rhizopus nigricans* The female race produced fumaric acid from a nutrient glucose solution, while the male race failed to produce fumaric acid under the same and a variety of conditions These results were not characteristic of sexual pairs in general

Ordinarily, molds produce only small quantities or traces of the acid, but at least one strain of *R nigricans* has the ability to convert 40 to 50 per cent of the sugar consumed to fumaric acid.

Other acids, for example, lactic acid, are frequently produced simultaneously with fumaric acid (see Chap XXXI).

Considerable information concerning the fumaric acid fermentation may be obtained through a study of the papers cited at the end of this chapter Birkinshaw[4] (1937) and Lockwood and Moyer[5] (1938) have very briefly reviewed some of the literature concerning the production of fumaric acid by molds Foster and Waksman have carried out extensive research concerning fumaric acid production by the genus *Rhizopus*, with special reference to the effect of zinc on growth and acid production.

In Table 130, some data concerned with molds that produce fumaric acid are summarized.

[1] FOSTER, J W, and S A WAKSMAN, *Jour Am. Chem Soc*, 61: 127 (1939)

[2] EHRLICH, F, *Ber*, 44: 3737 (1911)

[3] FOSTER, J W, and S A WAKSMAN, *Science*, 89: 37 (1939)

[4] BIRKINSHAW, J H, *Biol Rev*, 12: 357 (1937)

[5] LOCKWOOD, L B, and A J MOYER, *Bot Rev*, 4: 140 (1938)

Table 130 —Some Molds Producing Fumaric Acid[1]

Molds	Investigators	Year research reported
Aspergillus fumaricus	Wehmer[21,22]	1918, 1928
A. fumaricus	Thies[23]	1930
Circinella sp	Foster and Waksman[9]	1939
Cunninghamella sp	Foster and Waksman[9]	1939
Mucor sp	Foster and Waksman[9]	1939
M. stolonifer (R. nigricans)	Ehrlich[6,7]	1911, 1919
M. stolonifer (R. nigricans)	Butkewitsch & Federoff[3,4,5]	1929, 1930
M. stolonifer (R. nigricans)	Gottschalk[11]	1926
Penicillium griseo-fulvum Dierckx	Rasstrick & Simonart[14]	1933
Rhizopus japonicus	Takahaski, Sakaguchi, and Asai[17]	1926, 1927
R. nigricans	Waksman[20a]	1943
R. nigricans	Kane, Finlay & Amann[11a]	1943
R. niveus	Takahashi and Sakaguchi[16]	1925
R. oryzae	Ward, Lockwood, May, and Herrick[19]	1936
R. oryzae	Lockwood, Ward, & May[12]	1936
R. pseudochinensis	Takahashi and Asai[15]	1925
R. shanghaiensis	Takahashi and Sakaguchi[16]	1925
R. tritici	Takahashi and Sakaguchi[16]	1925

[1] The figures given in the parentheses refer to the bibliography on pages 628 and 629.

Waksman Process.—Waksman patented a process for producing fumaric acid from various carbohydrates and carbohydrate-containing materials, such as monosaccharides, molasses, and starch, by the use of selected fungi, particularly of the order Mucorales. Although strains of species of Cunninghamella, Circinella, and Rhizopus may be employed, strains of R. nigricans are preferred

The mold may be grown and the acid produced in a single medium, but preferably the mold is grown in one stage in a special medium and under conditions designed to encourage growth, and the acid is produced in a second stage through the use of one or more replacement media under conditions that stimulate acid production. Growth and acid production may take place by stationary surface culture methods or by submerged culture methods.

The growth medium contains sources of carbohydrate, nitrogen, nutrient salts, and trace elements or catalytic agents. A 5 to 15 per cent concentration of carbohydrate may be employed. Ammonium sulphate, salts of ammonia, and urea are satisfactory nitrogen sources but ammonium sulphate is preferred. A concentration of 0 2 per cent $(NH_4)_2SO_4$ is suitable, although the concentration of nitrogen may range from 0 2 to 0 5

g. per liter of medium. Additional nutrient salts may be supplied as 0.05 per cent of $MgSO_4 \cdot 7H_2O$ and 0.05 per cent of K_2HPO_4.

Certain elements or salts are used by Waksman to stimulate mold growth or fumaric acid production. Zinc, as $ZnSO_4 \cdot 7H_2O$, is added to the culture medium in concentrations of 1 to 10 mg. per liter of solution containing 50 to 150 g. of carbohydrate to encourage rapid formation of the mycelium. In order that growth may be followed at once by the maximum production of fumaric acid, the effect of the zinc is balanced with a salt of iron, for example $(Fe)_2(SO_4)_3$, in a concentration of 1 to 20 mg. per liter. Zinc and iron are associative in their effects when the ratio of zinc to iron is about 1 to 2, also when the initial concentration of carbohydrate is 20 to 30 per cent. Traces of manganese and copper may be added to the culture medium to accentuate the effect of the zinc.

The medium is sterilized with heat and inoculated with spores, or a suspension of germinated spores, of a selected strain of *R. nigricans*. The seeded medium is incubated at 28 to 35°C. to encourage growth of the mold. The latter is allowed to grow as a pellicle on the surface of the culture medium, or it is grown submerged in the medium while being agitated by aeration or mechanically at atmospheric or increased air pressures. A lattice-like support is recommended for surface culture of the mold, which requires 2 to 7 days. Submerged cultures require 24 to 60 hr for growth. The fumaric acid formed during the growth phase is neutralized with calcium carbonate or other alkali. At the completion of this phase, the growth solution is removed from the mycelium by draining, siphoning, or centrifuging, and saved for fumaric acid recovery.

During the second stage (replacement phase), a solution of carbohydrate (up to 20 per cent in concentration), not containing supplementary nitrogen or nutrient mineral salts, is added to the mold mycelium. Sufficient calcium carbonate to neutralize the fumaric acid expected (up to approximately 50 per cent of the carbohydrate) may be added or the acid may be neutralized periodically with KOH or NaOH to a pH of 5 to 6.5. Substances to stimulate fumaric acid formation (accelerators) are added to the replacement medium. For example, 0.1 to 1.0 g. each of $(Fe)_2(SO_4)_3$ and of $MgSO_4 \cdot 7H_2O$ may be added to 1 liter of solutions containing up to 20 per cent of carbohydrate, or 0.3 to 1 g. each of K_2HPO_4 and $MgSO_4 \cdot 7H_2O$ may be added to solutions containing about 200 g. of carbohydrate per liter. The solutions are incubated at 28°C. for 1 to 7 days.

Provision of a surface support for the mycelium in the case of stationary surface-culture fermentation and of aeration, agitation, and air under pressure in the case of submerged-culture fermentation accelerates the production of fumaric acid.

The replacement of the carbohydrate solution may be repeated until the enzyme system of the mold is impaired.

Kane-Finlay-Amann Process.—A process for producing fumaric acid, or a salt of this acid, with traces only of other acids has been patented by Kane, Finlay, and Amann (1943). The mold used is a strain of the genera *Mucor*, *Aspergillus*, or *Rhizopus*. A selected strain of *R. nigricans* is particularly suitable.

Fumaric acid is produced by submerged-culture methods in a medium containing a carbohydrate, nutrient salts, and a neutralizing agent. Glucose, fructose, invert sugar, sucrose, maltose, molasses, syrups, starches, or other materials may serve as the carbohydrate source. Nutrient salts may be supplied as potassium acid phosphate and ammonium sulphate. The medium may be mechanically agitated, aerated, or both, at atmospheric or elevated pressures. An oxygen-containing gas may be substituted for air. One illustration of the process follows:

A 30.25-lb. amount of hydrated glucose and small quantities of nutrient salts were diluted with water to make 50 gal. of solution. To this 20 lb. of calcium carbonate were added. The mixture was sterilized by boiling, cooled and seeded with 3 liters of a selected culture of *R. nigricans*. The fermentation medium was stirred at 40 r.p.m., while a stream of air was passed through it. Incubation was at a temperature of 28 to 32°C. The fermentation was stopped after 3 days and the liquor analyzed. A yield of 12 5 lb of fumaric acid was obtained.

References on Fumaric Acid

1. BERNHAUER, K , und H THIOLE: Über die Säurebildung durch Rhizopusarten I Die Bildung von Äpfelsäure bei der Fumarsäuregärung, *Biochem Zeit*, 287: 167–171 (1936).

2. BIRKINSHAW, J. H . Biochemistry of the Lower Fungi, *Biol Rev*, 12: 357 (1937).

3. BUTKEWITSCH, W., und M. W FEDEROFF: Über Bildung von Fumarsäure in den Zuckerculturen von *Mucor stolonifer* (*Rhizopus nigricans*) und sein Verhalten zur Brenztraubensäure, *Biochem Zeit*, 206: 440–456 (1929).

4. ——— und ———: Über die Umwandlung der Essigsäure durch *Mucor stolonifer* in Bernstein- und Fumarsäure und das Verfahren zur Trennung und quantitativen Bestimmung dieser Säuren, *Biochem Zeit*, 207: 302–318 (1929).

5. ——— ——— Über die Verhältnisse zwischen Essig-, Bernstein-, Fumar- ... Pilzen, ... 44: ⁹

6. EHRLICH, F.: Über die Bildung von Fumarsäure durch ..... 3737–3742 (1911).

7. ——— : Über Fumarsäure-Gärung des Zuckers, *Ber*, 52: 63–64 (1919).

8. FOSTER, J. W., and S. A. WAKSMAN Fumaric Acid Formation Associated with Sexuality in a Strain of *Rhizopus nigricans*, *Science*, 89: 37 (1939).

9. ——— and ———. The Production of Fumaric Acid by Molds Belonging to the Genus *Rhizopus*, *Jour Am Chem Soc*, 61: 127 (1939).

10. ⸻ and ⸻: The Specific Effect of Zinc and Other Heavy Metals on Growth and Fumaric-acid Production by *Rhizopus*, *Jour. Bact.*, 37: 599 (1939).

11. GOTTSCHALK, A : Biochemische Synthese von Fumarsäure aus Brenztraubensäure, *Zeit. physiol. Chem ,* 162: 136–143 (1926); 172: 314 (1927); 182: 311 (1929).

11a KANE, J H , A FINLAY, and P F. AMANN: Production of Fumaric Acid, U.S. Patent 2,327,191, Aug 17, 1943.

12 LOCKWOOD, L. B., G. E. WARD, and O. E. MAY: The Physiology of *Rhizopus oryzae*, *Jour. Agr. Research*, 53: 849–857 (1936).

13. ⸻ and A J MOYER· The Production of Chemicals by Filamentous Fungi, *Bot Rev*, 4: 140 (1938)

14. RAISTRICK, H , and P SIMONART: XXIX 2 5-Dihydroxybenzoic Acid (Gentisic Acid) A New Product of the Metabolism of Glucose by *Penicillium griseofulvum*, Dierckx, *Biochem Jour*, 27: 628–633 (1933),

15. TAKAHASHI, T , and T ASAI: On the Products of Fermentation by Mucor Group. II. The Products of Fermentation in Presence of Ca-carbonate *Centr. Bakt. Parasitenk ,* Abt II, 89: 81–84 (1933)

16. ⸻ and K SAKAGUCHI. Studies on the Acid Formed by *Rhizopus* Species, *Jour Agr Chem Soc (Japan)*, 1(5): 46 (1925).

17 ⸻, ⸻, and T ASAI. *Bull Agr Chem Soc (Japan)*, 2: 5 (1926), 3: 59 (1927)

18 THIES, W · Untersuchungen über den Einfluss der Bedingungen auf die Säurebildung des Schimmelpilzes *Aspergillus fumaricus*, *Centr Bakt. Parasitenk* Abt II, 82: 321–347 (1930).

19. WARD, G E, L B LOCKWOOD, O E MAY, and H T. HERRICK: Studies in the Genus *Rhizopus*. I The Production of Dextrolactic Acid, *Jour Am Chem Soc ,* 58: 1286–1288 (1936)

19a WAKSMAN, S A . Process for the Production of Fumaric Acid, U S Patent 2,326,986, Aug 17, 1943

20 ⸻, et J W FOSTER: Effect du zinc sur la végétation de *Rhizopus nigricans* et la production d'acide par cet organisme, *Compt rend ,* 207: 483 (1938)

21 WEHMER C Über Fumarsäure—Garung des Zuckers, *Ber ,* 51: 1663–1668 (1918)

22 ⸻ Abnahme des Säuregärungsvermogen und Anderung der Saure bei einem Pilz (Gluconsaure statt Fumarsäure—Gärung), *Biochem. Zeit.,* 197: 418–432 (1928)

THE GALLIC ACID FERMENTATION

Gallic acid (trihydroxybenzoic acid) has the following structural formula:
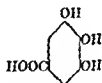
It occurs naturally in gall nuts, sumac, walnuts, tea, and other plants. It is prepared chemically by the hydrolysis of tannin.

Scheele discovered gallic acid when he was studying the effect of a mold on a water infusion of gall nuts (1787, or earlier). Gall nuts are rich in tannin compounds and are produced principally on species of the oak tree and the sumac, as the result of insect injuries.

Van Tieghem (1867) carried out classical studies in connection with the gallic acid fermentation. (The interested reader should refer to his early publications. To Van Tieghem, credit is due for being the first man to establish the importance of the aspergilli in the biochemical field, his work having been done at a time when very little was known of modern pure-culture methods.) Van Tieghem identified *Aspergillus niger* as the predominating mold in the fermentation. The *Penicillium glaucum* group of molds also possessed the ability to ferment tannin to gallic acid. Van Tieghem showed that air was essential for mold growth and for the successful production of gallic acid from moist gall nuts or tannin liquor.

In one of the oldest methods used for the production of gallic acid by fermentation, the substances containing tannin were piled up in heaps and moistened with water. Molds developed throughout the heap, which was stirred occasionally and maintained at a temperature of approximately 30°C. After a fermentation period of about a month the gallic acid was leached from the heap.

Present-day methods make use of clear tannin extracts, which are sterilized and then inoculated with pure cultures of a species of *Aspergillus*. The solution is agitated by mechanical means, and air is blown through it. The temperature of the fermentation is carefully controlled. Analysis of the mash is made occasionally in order that the fermentation

may be stopped promptly after the tannin has been completely utilized.

The conversion of tannin to gallic acid is actually brought about by means of the enzyme "tannase." Fernbach and Pottevin, each working independently, demonstrated that *A. niger* produced tannase in the presence of tannin and nutrient materials. Using the mold-free enzyme, it was shown that the fermentation would proceed independently of the mold.

Knudson discovered that he could cause a progressive increase in the tannase content of *A. niger* by replacing the sugar in Czapek's solution with tannic acid. He obtained a maximum production of tannase when the 10 per cent of sugar in the solution had been replaced with 2 per cent of tannic acid.

Gallic acid has several important uses in industry. It has been used in the manufacture of gallocyanin, a dye; it is used as a basic material in the production of alizarin brown. Inks are made from it. Condensation with sulphuric acid yields hexahydroxyanthraquinone. In combination, gallic acid finds use as a skin remedy.

References on the Gallic Acid Fermentation

CALMETTE, A Verfahren zur Umwandlung von Tannin in Gallussäure, German Patent 129164, Mar 1, 1902

DUCLAUX, E · Chimie biologique, in E Frémy, Encyclopédie Chimique, t 9, sect 1, Paris, 1883

FERNBACH, A Sur la tannase, *Compt rend*, **131**: 1214–1215 (1900)

KNUDSON, L Tannic Acid Fermentation, *Jour Biol Chem*, **14**: 159–202 (1913).

MAY, O E, and H T HERRICK: Some Minor Industrial Fermentations, *Ind Eng Chem* **22**: 1172–1176 (1930)

—— and —— Production of Organic Acids from Carbohydrates by Fermentation, *U S Dept Agr*, *Circ* 216, May, 1932

PORTER, J R "Bacterial Chemistry and Physiology," John Wiley & Sons, Inc., New York, 1946

POTTEVIN, H · La tannase, diastase dedoublant l'acide gallotannique, *Compt rend*, **131**: 1215–1217 (1900)

SCHEELE, K Über das Wesentliche Gallapfelsalz, *Crell's Chem Ann*, 1: 3–12 (1787)

THOM, C, and M B CHURCH: "The Aspergilli," The Williams & Wilkins Company, Baltimore, 1926

VAN TIEGHEM, P E L Fermentation gallique, *Ann sci nat Botan*, s 5, t 8, p 240 (1867)

——· Sur la fermentation gallique, *Compt rend*, **65**: 1091–1094 (1867)

THE ITACONIC ACID AND ITATARTARIC ACID FERMENTATIONS

THE ITACONIC ACID FERMENTATION

Itaconic acid, or methylene succinic acid, has the following structural formula:

$$CH_2$$
$$\overset{\|}{C}\cdot COOH$$
$$H_2C\cdot COOH$$

It is an unsaturated dibasic acid, which may be used for the preparation of resins and surface-active agents (such as detergents), or in the manufacture of synthetic organic chemical compounds. Its esters may be polymerized.

The first reports on the production of itaconic acid by mold fermentation were made by Kinoshita,[1] who stated that itaconic acid and mannitol were the principal products formed from sucrose by an organism that he designated as *Aspergillus itaconicus* Kinoshita. Later (1939) Calam, Oxford, and Raistrick[2] announced that a strain of *A. terreus* Thom produced significant yields of itaconic acid from glucose. However, other strains of the same species failed to produce this acid.

Extensive research on laboratory and semi-pilot-plant scales has been carried out by the Fermentation Division of the Northern Regional Research Laboratory of the U.S. Department of Agriculture.[3-6] This division was interested in developing a new industrial fermentation for the increased utilization of agricultural crops.

Organisms Employed.—Itaconic acid may be produced by certain strains of : . . . .[7] found that only . . .

[1] KINOSHITA, K., *Jour. Chem. Soc. Japan*, **50**: 583 (1929); *Acta Phytochim.* (Japan), **5**: 271 (1931); and **9**: 159 (1937).

[2] CALAM, C. T., A. E. OXFORD, and H. RAISTRICK, *Biochem. Jour.*, **33**: 1488 (1939).

[3] LOCKWOOD, L. B., and M. D. REEVES, *Arch. Biochem*, **6**: 455 (1945).

[4] MOYER, A. J., and R. D. COGHILL, *Arch. Biochem*, **7**: 167 (1945).

[5] LOCKWOOD, L. B., and G. E. WARD, *Ind. Eng. Chem*, **37**: 405 (1945).

[6] LOCKWOOD, L. B., and G. E. N. NELSON, *Arch. Biochem.*, **10**: 365 (1946).

[7] CALAM, OXFORD, and RAISTRICK, *loc. cit.*

itaconic acid from a Czapek-Dox medium containing 5 per cent glucose and 0.2 per cent potassium nitrate. Moyer and Coghill[1] surveyed 30 strains of this mold for itaconic acid production and obtained "promising yields" from one only. This strain, designated as *A. terreus* NRRL 265, produced 28 to 29 g of itaconic acid per 100 g of glucose in 10 to 12 days under optimum conditions. Lockwood and Reeves[2] surveyed 308 strains of *A. terreus*, which had been isolated by Raper and Alexander, of the Northern Regional Research Laboratory, and found 11 that gave yields from glucose greater than 45 per cent of the theoretical. One of the best of these, *A. terreus* NRRL 1960, was used in the researches of Lockwood and Reeves,[2] Lockwood and Ward,[3] and Lockwood and Nelson.[4]

Characteristics of ultraviolet-induced mutations of *A. terreus* have been described by Raper and coworkers[5] and by Lockwood and his associates.[6]

Stock Cultures.—These may be carried on slants containing Czapek-Dox solution agar

Methods of Production.—Itaconic acid may be produced by surface-culture or submerged-culture methods

Production by Surface-culture Methods.—Most of the methods reported in the literature are concerned with the production of itaconic acid by surface-culture methods.

Sporulation Media.—The production of large crops of spores is necessary for the inoculation of flasks and pans. These are obtained by the use of special media under the conditions specified.

Moyer and Coghill[7] used a liquid sporulation medium of the following composition.

Lactose	10 0 g	Corn-steep liquor	5 0 ml
Glucose	5 0 g	Fe tartrate	0 005 g
KH₂PO₄	0 060 g	CuSO₄ 5H₂O	0 004 g
MgSO₄ 7H₂O	0 050 g	MnSO₄ 4H₂O	0 005 g
KCl	0 100 g	Agar	0 100 g
NaCl	5 00 g	Distilled water to	1 0 liter
KNO₃	3 00 g		

[1] Moyer, A J , and R D Coghill, *Arch Biochem*, **7:** 167 (1945).

[2] Lockwood, L B , and M D Reeves, *Arch Biochem*, **6:** 455 (1945)

[3] Lockwood, L B , and G E Ward, *Ind Eng Chem*, **37:** 405 (1945)

[4] Lockwood, L B , and G. E. N Nelson *Arch Biochem*, **10:** 365 (1946)

[5] Raper, K B , R D Coghill, and A Hollaender, *Am Jour. Bot* **32** (No 3): 165–176 (1945)

[6] Lockwood, L B , K B Raper, A. J Moyer, and R. D Coghill, *Am Jour Bot* **32** (No 4) 214–217 (1945)

[7] Moyer and Coghill, *loc cit*

The medium was dispensed in 200-ml. Erlenmeyer flasks in amounts of 40 ml. each and sterilized. Good yields of spores were obtained in 5 to 7 days at 27°C.

In another type of sporulation medium described by Moyer and Coghill,[1] 25 g. of wheat bran were moistened with 20 ml. of the medium described above. The moistened wheat bran was placed in 750-ml. Erlenmeyer flasks and sterilized. The cooled product was then inoculated with spores and incubated for 5 to 7 days, during which time a heavy crop of spores was produced. The bran was allowed to dry and 2 volumes of sterile whole wheat flour were added and thoroughly mixed by means of a sterile spatula. According to Moyer and Coghill, about 0.200 g. of this spore-containing mixture was adequate for seeding a 200-ml. Erlenmeyer flask and about 0.5 g. was sufficient for a 3-liter Fernbach flask.

Lockwood and Ward used a sporulation medium of the following composition in the semi-pilot-plant scale production of itaconic acid.

Glucose monohydrate (commercial)	. . . .	275 g.
NaNO₃	.	5 g
MgSO₄·7H₂O		0 024 g
KCl		0 005 g.
H₃PO₄		0 003 g
Concentrated corn-steep liquor		0 5 ml.
Distilled water to		1,000 ml

The medium was dispensed in 200-ml. Erlenmeyer flasks in 50-ml. portions and sterilized. The spores from a 10-day-old slant culture were then used to seed each flask heavily. The flasks were incubated at 30°C. for 5 days, which was ample time for the production of an abundance of spores Lockwood and Ward stated that the culture in one flask was sufficient to inoculate 100 liters of fermentation medium.

Factors Affecting Production.—Basic research concerning the factors affecting the production of itaconic acid by surface-culture methods has been carried out by Lockwood and Reeves, and by Moyer and Coghill, of the U.S. Department of Agriculture, and by others. Some of the findings of the U.S. Department of Agriculture group will now be reviewed.

CARBOHYDRATE SOURCE.—Concentrations of glucose ranging from 20 to 25 per cent appeared to produce the highest yields of itaconic acid on a laboratory scale. In their investigations, Moyer and Coghill used either 75 ml of medium containing 20 per cent glucose or 60 ml. of medium containing 25 per cent glucose in each 200-ml. Erlenmeyer flask.

The presence of unfermented glucose interfered with the recovery of the itaconic acid.

[1] MOYER and COGHILL, *loc cit.*

NITROGEN SOURCES.—Nitrogen was supplied to the medium through the use of a nitrogen-containing salt, nitric acid, and corn steep liquor.

Moyer and Coghill found that ammonium nitrate was the best source of nitrogen for itaconic acid production by *A terreus* NRRL 265. Ammonium chloride was not as good as ammonium nitrate but was markedly superior to diammonium hydrogen phosphate, sodium nitrate, potassium nitrate, and urea. Ammonium sulphate also was inferior to ammonium nitrate Potassium nitrate and sodium nitrate produced the heaviest mold growth, but poor yields of itaconic acid.

Nitric acid, which was used to adjust the reaction of the medium to a favorable range, also contributed nitrogen

Corn-steep liquor was used as a source of soluble protein derivatives, trace elements and other minerals, as well as growth-promoting substances which enhanced the fermentation According to Bowden and Peterson,[1] samples of corn-steep liquor usually contain 40 to 60 per cent of solids On a dry basis, they contain 12 to 27 per cent of lactic acid, 7.4 to 7 8 per cent of total nitrogen, 2.6 to 3.3 per cent of amino nitrogen, 1.5 to 14 per cent of reducing sugars (calculated as glucose), and 18 to 20 per cent of ash.

In a concentration of 0 2 per cent, corn-steep liquor decreased the time required for the germination of the mold spores and also increased the rate of growth of the mycelium during the first part of the fermentation.

The optimum concentration of the concentrated corn-steep liquor appeared to be 0 4 per cent by volume (4 ml per liter). Larger concentrations favored heavier growth of mycelia but lower yields of itaconic acid

MAGNESIUM SULPHATE—The effect of this salt on the production of itaconic acid was so striking that it will be given detailed consideration here A higher concentration of magnesium sulphate was used in the production of itaconic acid than is employed in the usual mold fermentation The optimum amount found for the *A terreus* NRRL 1960 fermentation appeared to be 4 4 to 5 g of $MgSO_4 \cdot 7H_2O$ per liter of medium.[2] The magnesium sulphate served not only as the usual required nutrient but also as a stimulant for the production of itaconic acid and for increasing the acid tolerance of the mold For example, growth and fermentation occurred at a pH of 1 4 when the concentration of $MgSO_4 \cdot 7H_2O$ was 4 75 g per liter of medium but no growth occurred at this pH when the concentration of $MgSO_4 \cdot 7H_2O$ was 0 25 g per liter Magnesium sulphate also tended to counteract the toxicity due to aluminum ions (see following page).

[1] BOWDEN, J P, and W H PETERSON, *Arch Biochem*, 9: 387 (1946).
[2] LOCKWOOD and REEVES, *loc cit*

ROLE OF OTHER SALTS.—Iron, as ferric tartrate, increased the acid accumulation by *A. terreus* NRRL 1960, when present in a concentration of 5 mg. per liter, particularly at a pH of 2.0. Iron, or zinc, or both iron and zinc, resulted in marked increases of itaconic acid accumulation at a pH of 2.0, but there was no advantage to be gained from adding both. When a pH of 3.0 was employed, the addition of 1 mg. or more of zinc per liter and even of 10 mg. or more per liter resulted in a marked increase in growth but no accumulation of itaconic acid. With an initial pH of 1.7, itaconic acid accumulated faster with a high iron concentration than with a high zinc concentration. In the media advocated by Lockwood and Reeves and Lockwood and Ward[1] for *A. terreus* NRRL 1960, zinc sulphate was used in a concentration of 0.0044 g. per liter, but no iron was employed.

Metallic aluminum and aluminum sulphate were toxic to a number of strains of *A. terreus*. Strips of aluminum, when placed in media, prevented the growth of several strains of this organism. Aluminum sulphate in a concentration equivalent to 10 mg. per liter of aluminum ions prevented the growth of the mold at a pH of 2.0; and in a concentration equivalent to 20 mg per liter of aluminum ions it permitted only a slight amount of growth at a pH of 3.0. Magnesium sulphate in the optimum concentration found (4.4 to 5.0 g. per liter) counteracted the apparent toxicity due to the aluminum ions.

The use of an excess of potassium chloride, or of potassium, sodium, chloride, or sulphate ions, resulted in lower yields of itaconic acid. The potassium and sodium ions tended to raise the pH, while the chloride and sulphate ions were inclined to lower the pH when present in sufficient concentration

Sodium chloride was sometimes used instead of potassium chloride in the medium for producing itaconic acid. The usual concentration of KCl was 0 050 g. per liter, while that of NaCl was 0 4 g. per liter

Manganese, molybdenum, copper, cobalt, nickel, chromium, gallium, and borate ions, each in concentrations of 1, 10, 20, 50, or 100 mg. of the desired ions per liter, failed to increased yields of itaconic acid or mycelium at initial pH values of 2.0, 2.5, and 3 0.

pH.—In order to obtain a good yield of itaconic acid, it is necessary to adjust the pH to a low initial value and to maintain it within a relatively narrow range during the fermentation. The optimum initial pH for itaconic acid accumulation lies within the pH range of 1.9 to 2.3. Below pH 1.9 production of mycelium is seriously retarded, which results in low yields of itaconic acid. As the pH is increased to about 2.7, the amount of growth increases to a maximum (as measured by the weight of the

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[1] LOCKWOOD and WARD, *loc. cit.*

mycelium); however, this is not true of acid accumulation for this appears to be at a peak at a pH of 2.1 to 2.2. Thus the optimum pH for acid production is lower than that required for producing optimum mycelial growth.

It is to be repeated that the addition of 4 75 g. of $MgSO_4 \cdot 7H_2O$ to each liter of medium increased the tolerance of the mold to acid, as was indicated by the fact that it would grow and produce itaconic acid at an initial pH of 1 4 when this amount of the salt was present.

Itaconic acid was metabolized by *A. terreus* NRRL 1960, particularly when the pH was above 2.3. Thus the need for maintaining the lowest pH value consistent with adequate growth and maximum acid accumulation is evident.

Nitric acid is preferred for adjusting the pH of the medium since it enhances itaconic acid production As noted above, it also supplies nitrogen to the mold. The medium used for the semi-pilot-plant scale production of itaconic acid contains 1.60 ml of nitric acid (of specific gravity, 1.42) per liter of nutrients.

TEMPERATURE.—The optimum temperature for the production of itaconic acid by the strains studied appears to be 30°C.

DURATION OF FERMENTATION —A period of 10 to 12 days is usually required for the completion of fermentation by surface-culture methods.

TABLE 131—COMPOSITION OF MEDIA USED FOR ITACONIC ACID PRODUCTION

Ingredient	Medium of Lockwood and Reeves[1] amount per liter	Medium of Moyer and Coghill,[2] amount per liter
Glucose	250 0 g	250 0 g
$MgSO_4 \cdot 7H_2O$	4 5 g	0 25 g
NH_4NO_3	2 5 g	2 0 g
KCl		0 050 g
NaCl	0 4 g	
$ZnSO_4 \cdot 7H_2O$	0 0044 g	0 014 g
HNO_3 (N/2).		50 ml.
HNO_3 (sp gr 1.42) .	1 60 ml	4 0 ml
Corn steep liquor	4 0 ml	4 0 ml
Dist. water to	1,000 0 ml	1,000 0 ml

[1] *Arch Biochem.,* **6** (No 3) 455 (1945)
[2] *Arch Biochem.,* **7** (No 1) 167 (1945)

Composition of Production Media.—The composition of media found to be about optimum for itaconic acid production in the laboratory is given in Table 131

Production on a Semi-pilot-plant Scale.—A process for producing itaconic acid on a semi-pilot-plant scale was described by Lockwood and Ward.[1] The mold used was *A. terreus* NRRL 1960. Spores for the inoculation of the fermentation medium were produced on the sporulation medium described on p. 634. The fermentation medium employed by them had the following constituents:

Glucose monohydrate (commercial)	... ...	165 g
MgSO₄7H₂O ..	... ...	4.4 g
NH₄NO₃	.. ..	2.5 g
NaCl	. . ..	0.4 g
ZnSO₄·7H₂O	. . .	0.0044 g.
Nitric acid (sp. gr. 1.42)	. . .	1.60 ml
Concentrated corn steep liquor		4.0 ml
Distilled water to.		1,000 ml

The medium was sterilized and seeded with mold spores after cooling Twelve-liter portions of the inoculated medium were introduced aseptically into each of a series of sterilized shallow aluminum pans (22 × 36 × 2 in.) contained in a special incubator cabinet.

During the fermentation, the temperature was maintained at 30 to 32°C. by means of a thermostat that regulated the flow of water through the coils contained in the cabinet. Sterile humidified air was passed

TABLE 132 —PRODUCTION OF ITACONIC ACID FROM GLUCOSE[1]

	A. Typical pan fermentations		B. Duplicate pan fermentations at initial glucose concentrations of;			
	Pan 1	Pan 2	10 per cent	15 per cent	20 per cent	25 per cent
Initial glucose, grams (calcd to anhydrous basis)	1800	1800	1200	1800	2400	3000
Glucose consumed, grams	1583	1539	1128	1565	1617	1810
Itaconic acid produced, grams	569	602	242	561	577	568
Yield of itaconic acid based on glucose consumed,[2] per cent	49.9	54.3	29.8	49.7	49.5	43.5
Itaconic acid recovered by crystallization, grams	453	490	192	436	457	429
Recovery efficiency, per cent	79.7	81.4	79.4	77.8	79.2	75.2
Recovery weight yield,[3] per cent	25.2	27.2	16.0	24.2	19.0	14.3
Mycelial weight, grams	222	144	223	233	245	277

[1] Lockwood, L. B., and G. E. Ward, *Ind. Eng. Chem.*, **37** 405 (1945)

[2] Based on assumption that 1 mole of glucose should yield 1 mole of itaconic acid

[3] Ratio of grams itaconic acid recovered to grams glucose supplied (anhydrous basis)

[1] Lockwood and Ward, *loc. cit.*

through the cabinet at the rate of 5 liters per min. to aerate the molds. The fermentation was generally completed in 12 days.

Table 132 shows the effects on typical semi-pilot-plant scale yields when the initial glucose concentrations were varied.

Production in Submerged Culture.—In research carried out on a laboratory scale, Lockwood and Nelson[1] determined the optimum conditions for the accumulation of itaconic acid in submerged cultures: *i.e.*, the proper quantity of inoculum, pH, and concentrations of magnesium sulphate, sodium chloride, zinc sulphate, corn steep liquor, and glucose.

Organism Used—The mold used was *A. terreus* NRRL 1960

Fig. 88—The effect of pH on the growth and acid production of *A. terreus* NRRL 1960
[*Courtesy of L. B. Lockwood and G. E. N. Nelson, Arch. Biochem., 10 (No. 3): 365 (1946).*]

Preparation of Inoculum—Pellets were used as the inocula of the fermentation medium. These were prepared by inoculating a sterile medium (containing 50 g. of glucose, 2.5 g. of NH_4NO_3, 0.6 g. of KH_2PO_4, 0.25 g. of $MgSO_4 \cdot 7H_2O$, and 4 ml. of corn steep liquor per liter of solution) with dry spores from either an agar-slant or unagitated liquid culture of *A. terreus*, and then agitating it on a platform shaker for 48 hr. with incubation at 30°C. The optimum quantity of inoculum for 125 ml. of medium in a 300-ml. Pyrex Erlenmeyer flask was 1 to 2 pellets of about 2-mm. diameter each.

Optimum Conditions for Production—Lockwood and Nelson[2] found the following conditions to be optimum for submerged-culture production of itaconic acid at 30°C.

pH.—The optimum pH lay between 1.8 and 1.9. Rigid control of the pH was essential for highest yields of itaconic acid. Figure 88 shows that

[1] LOCKWOOD and NELSON, *loc. cit.*
[2] *Ibid.*

Production on a Semi-pilot-plant Scale.—A process for producing itaconic acid on a semi-pilot-plant scale was described by Lockwood and Ward.[1] The mold used was *A. terreus* NRRL 1960 Spores for the inoculation of the fermentation medium were produced on the sporulation medium described on p. 634. The fermentation medium employed by them had the following constituents:

Glucose monohydrate (commercial)	165 g
MgSO₄·7H₂O	4 4 g
NH₄NO₃	
NaCl	2 5 g
ZnSO₄·7H₂O	0.4 g
Nitric acid (sp. gr. 1 42)	0.0044 g.
Concentrated corn steep liquor	1.60 ml.
Distilled water to	4.0 ml.
	1,000 ml

The medium was sterilized and seeded with mold spores after cooling.

Twelve-liter portions of the inoculated medium were introduced aseptically into each of a series of sterilized shallow aluminum pans (22 × 36 × 2 in.) contained in a special incubator cabinet.

During the fermentation, the temperature was maintained at 30 to 32°C. by means of a thermostat that regulated the flow of water through the coils contained in the cabinet. Sterile humidified air was passed

TABLE 132.—PRODUCTION OF ITACONIC ACID FROM GLUCOSE[1]

	A. Typical pan fermentations		B Duplicate pan fermentations at initial glucose concentrations of:			
	Pan 1	Pan 2	10 per cent	15 per cent	20 per cent	25 per cent
Initial glucose, grams (calcd. to anhydrous basis)	1800	1800	1200	1800	2400	3000
Glucose consumed, grams	1583	1539	1128	1565	1617	1810
Itaconic acid produced, grams	569	602	242	561	577	568
Yield of itaconic acid based on glucose consumed,[2] per cent	49.9	54 3	29 8	49 7	49.5	43 5
Itaconic acid recovered by crystallization, grams	453	490	192	436	457	429
Recovery efficiency, per cent	79 7	81 4	79 4	77 8	79 2	75 2
Recovery weight yield,[3] per cent	25 2	27 2	16 0	24.2	19.0	14.3
Mycelial weight, grams	222	144	223	233	245	277

[1] LOCKWOOD, L B., and O E WARD, *Ind Eng Chem.*, **37**, 405 (1945).
[2] Based on assumption that 1 mole of glucose should yield 1 mole of itaconic acid.
[3] Ratio of grams itaconic acid recovered to grams glucose supplied (anhydrous basis)

[1] LOCKWOOD and WARD, *loc. cit.*

through the cabinet at the rate of 5 liters per min. to aerate the molds. The fermentation was generally completed in 12 days.

Table 132 shows the effects on typical semi-pilot-plant scale yields when the initial glucose concentrations were varied.

Production in Submerged Culture.—In research carried out on a laboratory scale, Lockwood and Nelson[1] determined the optimum conditions for the accumulation of itaconic acid in submerged cultures: i e , the proper quantity of inoculum, pH, and concentrations of magnesium sulphate, sodium chloride, zinc sulphate, corn steep liquor, and glucose.

Organism Used.—The mold used was *A. terreus* NRRL 1960.

FIG 88 —The effect of pH on the growth and acid production of *A terreus* NRRL 1960 [*Courtesy of L B Lockwood and G E N Nelson, Arch Biochem , 10 (No 3) 365 (1946)]*

Preparation of Inoculum —Pellets were used as the inocula of the fermentation medium. These were prepared by inoculating a sterile medium (containing 50 g of glucose, 2 5 g of NH_4NO_3, 0.6 g of KH_2PO_4, 0 25 g of $MgSO_4 \cdot 7H_2O$, and 4 ml of corn steep liquor per liter of solution) with dry spores from either an agar-slant or unagitated liquid culture of *A. terreus*, and then agitating it on a platform shaker for 48 hr. with incubation at 30°C. The optimum quantity of inoculum for 125 ml of medium in a 300-ml. Pyrex Erlenmeyer flask was 1 to 2 pellets of about 2-mm diameter each.

Optimum Conditions for Production.—Lockwood and Nelson[2] found the following to be optimum for submerged-culture production of itaconic acid at 30°C

pH.—The optimum pH lay between 1 8 and 1 9 Rigid control of the pH was essential for highest yields of itaconic acid Figure 88 shows that

[1] LOCKWOOD and NELSON, *loc cit*
[2] *Ibid*

Production on a Semi-pilot-plant Scale.—A process for producing itaconic acid on a semi-pilot-plant scale was described by Lockwood and Ward.[1] The mold used was *A. terreus* NRRL 1960. Spores for the inoculation of the fermentation medium were produced on the sporulation medium described on p. 634. The fermentation medium employed by them had the following constituents:

Glucose monohydrate (commercial)	165 g
MgSO$_4$·7H$_2$O	4.4 g.
NH$_4$NO$_3$	2.5 g.
NaCl	0.4 g
ZnSO$_4$·7H$_2$O	0.0044 g.
Nitric acid (sp. gr. 1.42).	1.60 ml
Concentrated corn steep liquor	4.0 ml.
Distilled water to.	1,000 ml

The medium was sterilized and seeded with mold spores after cooling.

Twelve-liter portions of the inoculated medium were introduced aseptically into each of a series of sterilized shallow aluminum pans (22 × 36 × 2 in.) contained in a special incubator cabinet

During the fermentation, the temperature was maintained at 30 to 32°C. by means of a thermostat that regulated the flow of water through the coils contained in the cabinet. Sterile humidified air was passed

TABLE 132.—Production of Itaconic Acid from Glucose[1]

	A. Typical pan fermentations		B. Duplicate pan fermentations at initial glucose concentrations of:			
	Pan 1	Pan 2	10 per cent	15 per cent	20 per cent	25 per cent
Initial glucose, grams (calcd to anhydrous basis)	1800	1800	1200	1800	2400	3000
Glucose consumed, grams	1583	1539	1128	1617	1810	1810
Itaconic acid produced, grams	569	602	242	561	577	568
Yield of itaconic acid based on glucose consumed,[2] per cent	49.9	54.3	29.8	49.7	49.5	43.5
Itaconic acid recovered by crystallization, grams	453	490	192	436	457	429
Recovery efficiency, per cent	79.7	81.4	79.4	77.8	79.2	75.2
Recovery weight yield,[3] per cent	25.2	27.2	16.0	24.2	19.0	14.3
Mycelial weight, grams	222	144	223	233	245	277

[1] Lockwood, L. B., and G. E. Ward, *Ind Eng Chem*, **37**, 405 (1945).

[2] Based on assumption that 1 mole of glucose should yield 1 mole of itaconic acid

[3] Ratio of grams itaconic acid recovered to grams glucose supplied (anhydrous basis).

[1] Lockwood and Ward, *loc. cit.*

tinuous agitation. The liquor from 1 pan (about 12 liters) is concentrated to approximately 1 liter. Crystals of itaconic acid form during the concentration process. The slurry is cooled to room temperature, after which the crystals are separated by centrifuging and washed with cold water. Further concentration of the fermentation liquor may result in a second crop of itaconic acid crystals. Ninety per cent recovery may usually be obtained by repeated crystallizations.

Determination of the Acid in Fermentation Liquors.—In the determination of the itaconic acid content of a fermentation liquor, an estimate may be obtained by titrating an aliquot sample with a standard alkali solution, using phenolphthalein as the indicator. In order that the resulting figure might be considered absolutely veracious, it would have to be assumed that itaconic acid was the only acid produced by the mold. That this is not necessarily the case has been shown by Calam, Oxford, and Raistrick,[1] who reported that several strains of *A. terreus* form fumaric, oxalic, and succinic acids. Hence, unless the amounts of these acids produced by the mold are known, titration gives an estimate only.

Friedkin[2] has developed a method (a modification of the Koppeschaar method[3]), for the direct determination of itaconic acid in fermentation liquors. This involves the measurement of bromine absorption by itaconic acid in acid-buffered bromine water and depends on the fact that aqueous bromine at a pH of 1.2 reacts equimolecularly with itaconic acid but does not react with glucose. The method is as follows:

One or two milliliters (usually 2 ml.) of a uniform sample of the fermentation liquor are introduced into a 125-ml. iodine flask by means of a pipette. To the sample are added 50 ml. of acid-buffered bromine water (at pH 1 2). The stopper of the iodine flask is then sealed with water to prevent the loss of bromine vapor. The flask is allowed to stand at room temperature for 10 min. and is then placed in an ice bath. After cooling for 5 min., 5 ml. of strong potassium iodide solution (made by dissolving 50 g of potassium iodide in 100 ml. of distilled water) are placed in the well surrounding the stopper of the iodine flask. The stopper is carefully lifted, and the potassium iodide solution is sucked into the flask as a result of the partial vacuum created by cooling the flask plus its contents.

After allowing 10 min. for the reaction, the iodine released from the potassium iodide is titrated with 0 1 N sodium thiosulphate, using starch as the indicator.

[1] Calam, Oxford, and Raistrick, *loc cit.*

[2] Friedkin, M, *Ind Eng Chem , Anal Ed*, 17: 637 (1945)

[3] Koppeschaar, W F, *Zeit anal Chem*, 15: 233 (1876)

effect of pH on mycelial weight and itaconic acid production by both submerged- and surface-culture methods. Usually 50 ml. of 0.5 N nitric acid were added per liter of medium.

CORN-STEEP LIQUOR CONTENT.—The optimum concentration was approximately 1.5 ml. per liter. Larger concentrations favored the development of heavier mycelium and less total itaconic acid.

SALT CONCENTRATIONS.—The optimum concentration of magnesium sulphate was about 0.75 g. per liter. The use of sodium chloride resulted in greater growth of mycelium and marked diminution in the yield of itaconic acid. Zinc sulphate did not increase the yields of acid when used as a constituent of the medium. Ammonium nitrate was used in a concentration of 2.5 g. per liter; and ferric tartrate (green) was generally used in a concentration of 0.15 g. (30 mg. of ferric ions) per liter.

GLUCOSE CONCENTRATION.—The optimum concentration, based on utilization and itaconic acid yield, was about 60 g. per liter. When larger concentrations were used, there were greater residual (or unused) amounts of glucose.

A patent has been issued to Kane, Finlay, and Amann[1] relating to the production of itaconic acid and its salts by selected fungus strains in submerged aerobic growth by a method stated to be usable. Itaconic acid-producing strains of *Aspergillus terreus* or of other molds, particularly of the genus *Aspergillus*, may be used.

An example of the process follows:[1] A 2-liter mash of diluted molasses, containing 196 g. of a mixture of sucrose and invert sugar, or of sucrose, 30 g. of sodium nitrate, and 1.0 g. of sodium alginate, was inoculated with 2.5 ml. of a suspension of the spores of a strain of *A. terreus* and incubated at 34 to 38°C. The mash was agitated vigorously by a "high-speed, propeller-type stirrer" which provided aeration. The fermentation was stopped after 7 days and 18 g. of itaconic acid were found.

In another case, a 2-liter mash containing 195 g. of sucrose, 42 g. of Ca(NO₃)₂·4H₂O, 1 g. of sodium alginate, and suitable sources of phosphorus, potash, and trace elements, was inoculated with 2.5 ml. of a mold spore suspension and incubated at 34 to 38°C. The mash was agitated and aerated. At the end of 2 weeks, the liquor contained 54 g. of itaconic acid.

Recovery of the Acid.—The spent solutions are drained or filtered from the pans or other containers into a suitable vessel for concentration. The mycelia and pans are washed with small amounts of cold water, and the washings thus obtained are combined with the main filtrate. The liquor is concentrated on a steam bath, or by other means, using con-

[1] KANE, J. H., A. C. FINLAY, and P. F. AMANN, U.S Patent 2,385,283, Sept 18 1945

procedure followed in isolating and identifying the products may be obtained by reference to the original report.[1]

References on Itaconic Acid

CALAM, C T , A E OXFORD, and H RAISTRICK: The Biochemistry of Microorganisms LXIII. Itaconic Acid, a Metabolic Product of a Strain of *Aspergillus terreus* Thom, *Biochem Jour* , 33: 1488–1495 (1939).

FRIEDKIN, M.: Determination of Itaconic Acid in Fermentation Liquors, *Ind Eng. Chem* , *Anal Ed* , 17: 637–638 (1945).

KANE, J H , A C FINLAY, and P F AMANN: Production of Itaconic Acid, U.S Patent, 2,385,283, Sept 18, 1945.

KINOSHITA, K : Formation of Itaconic Acid and Mannitol by a New Filamentous Fungus, *Jour Chem Soc Japan*, 50: 583–593 (1929)

———— Über die produktion von Itaconsäure und Mannit durch einen neuen Schimmelpilze, *Aspergillus itaconicus*, *Acta Phytochim (Japan)*, 5: 271–287 (1931)

————: Physiological and Biochemical Studies of *Aspergillus itaconicus*, *Acta Phytochim (Japan)*, 9: 159–187 (1937)

LOCKWOOD, L B , and G E N NELSON: Some Factors Affecting the Production of Itaconic Acid by *Aspergillus terreus* in Agitated Cultures, *Arch Biochem* , 10 (No 3): 365–374 (1946)

————, K B RAPER, A J MOYER, and R D COGHILL: The Production and Characterization of Ultraviolet-induced Mutations in *Aspergillus terreus* III Biochemical Characteristics of the Mutations, *Am. Jour. Bot* , 32 (No 4) 214–217 (1945).

———— and M D REEVES: Some Factors Affecting the Production of Itaconic Acid by *Aspergillus terreus*, *Arch Biochem* , 6: 455–469 (1945)

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STODOLA, F H , M FRIEDKIN, A J MOYER, and R D COGHILL Itatartaric Acid, a Metabolic Product of an Ultraviolet-induced Mutant of *Aspergillus terreus*, *Jour Biol Chem* , 161 (No 2) 739–742 (1945)

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[1] STODOLA, F H , M FRIEDKIN, A. J. MOYER, and R D COGHILL, *Jour. Biol. Chem* , 161 (No 2): 739–742 (1945)

A blank containing 50 ml. of bromine water is treated in the same manner as the sample of fermentation liquor.

The number of milliliters of 0.1 N sodium thiosulphate required to react with the iodine released from the potassium iodide by the 50-ml. blank of bromine water (X) minus the number of ml. of 0.1 N sodium thiosulphate required to react with the iodine released by the bromine that did not react with itaconic acid (Y) is equal to the number of milliliters of 0.1 N itaconic acid. Therefore, $(X - Y)0.1 =$ the number of milliequivalents of itaconic acid and $(X - Y)0.0065 =$ the weight in grams of the itaconic acid in the sample analyzed.

The reagent (bromine water) used in the analysis contains the following constituents:

Bromine . ..			1.0 ml.
Potassium bromide.	. .	. . . .	3 0 g.
Potassium chloride			. 1 87 g
1 0 N HCl		. .	48 5 ml
Water (distilled) to		. . . . .	500 ml.

The bromine and potassium bromide are dissolved in a small portion of distilled water and the other ingredients are then added. The resultant pH is 1.2 ± 0.1 and hence no further adjustment is necessary. The reagent should be stored in a dark bottle, preferably in a refrigerator.

According to Friedkin, the following substances, which may be found in fermentation liquors, do not interfere with the analysis within experimental accuracy: 15 per cent glucose, 1 N d-gluconolactone, 1 N acetic acid, 1 N aconitic acid, 1 N citric acid, saturated fumaric acid, 1 N lactic acid, 1 N malic acid, 1 N oxalic acid, 1 N succinic acid, and 1 N tartaric acid.

THE ITATARTARIC ACID FERMENTATION

In 1945, Stodola, Friedkin, Moyer, and Coghill, of the Northern Regional Research Laboratory, reported on the production of itatartaric acid by an ultraviolet-induced mutant of *Aspergillus terreus*. The mold was grown in a medium that contained 220 g. of glucose, 0.50 g. of $MgSO_4 \cdot 7H_2O$, 0.05 g. of KCl, 0.418 g of H_3PO_4, 0.022 g. of $ZnSO_4 \cdot 7H_2O$, 4.0 ml. of 0.50 N HCl, 8 0 ml. of 0.50 N HNO_3, 4 g. of NH_4NO_3, 1.0 g. of corn steep liquor, and distilled water to make 1 liter. The medium was dispensed in 750-ml. amounts in 3-liter flasks and inoculated with *A. terreus* NRRL 265 S14. The flasks were incubated at 30°C. for 10 days. From 3.75 liters of the culture liquor, after the removal of the mycelial mats, was obtained 134.9 g. of crystalline itaconic acid. A mixture of itatartaric acid and its lactone accounted for 5.8 per cent of the total weight of acid accumulated during the fermentation Details of the

procedure followed in isolating and identifying the products may be obtained by reference to the original report.[1]

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ability to produce kojic acid from sucrose. Among the molds mentioned were *A. oryzae*; *A. flavus*, var.; *A. gymnosardae*; *A. awamori*; *A. candidus*, *A. clavatus*; *A. fumigatus*; and *A. giganteus*.

In 1929, Challenger, Klein, and Walker[1] published the results of research which showed that kojic acid could be produced from xylose by *A. oryzae*. During the same year Katagiri and Kitahara[2] reported on the formation of kojic acid from pentoses, gluconic acid, and other substances

Since 1929, several papers have been published concerning various aspects of the kojic acid fermentation. Titles to some of the more significant papers will be found in the reference list at the end of the chapter.

Microorganisms Producing Kojic Acid.—Several molds of the genus *Aspergillus* have the ability to produce kojic acid from suitable carbon-containing nutrient solutions. In addition to the species enumerated in the foregoing paragraphs, the following molds produce kojic acid: *A. albus*, *A. effusus*, *A. nidulans*, *A. parasiticus*, *A. tamarii*, and *Penicillium dalece*. From among the bacteria, several species of *Acetobacter* may also form kojic acid under favorable conditions.

Carbon Sources.—A fairly large number of carbon-containing substances have been fermented with the production of kojic acid by different microorganisms. These substances include starches; dextrins; disaccharides, such as sucrose and maltose; the hexoses—glucose, fructose, mannose, and galactose; the pentoses—xylose and arabinose; and sorbitol, dulcitol, *i*-adonitol, inulin, inositol, glycerol, glycero-betn-phosphate, dihydroxyacetone, gluconic acid, tartaric acid, and other substances. The best yields have, in general, been obtained from glucose and xylose.

Concentration of Carbon-containing Substance Used.—The concentrations of carbon-containing materials used have been varied from approximately 5 to 30 per cent. May and his associates[3] used sugar concentrations varying from 15 to 33 per cent in their work. They obtained highest yields with *A. flavus* when using a concentration of 20 per cent glucose. Barham and Smits[4] found a 15 per cent concentration of xylose to be most suitable for fermentation. Katagiri and Kitahara used 5 per cent concentrations of a large number of substances with satisfactory results in most cases. The same concentration has been used by other workers.

[1] CHALLENGER, F., L KLEIN, and T. K. WALKER, *Jour Chem Soc*., p. 1498 (1929)

[2] KATAGIRI, H., and K. KITAHARA, *Bull Agr Chem Soc (Japan)*, 5: 38 (1929)

[3] MAY, O. E., A. J. MOYER, P. A. WELLS, and H. T. HERRICK, *Jour. Am. Chem Soc.*, 53: 771 (1931).

[4] BARHAM, H. N., and B. L. SMITS, *Ind Eng Chem*, 28: 567 (1936).

Nutrient Salts.—The types of nutrient salts used for kojic acid production are illustrated in the paragraphs following.

May and his coworkers[1] obtained satisfactory results when using the following salts in the concentrations recorded:

	Grams per Liter of Glucose Solution
$MgSO_4.7H_2O$	0 500
KCl	0 100
H_3PO_4	0 053
NH_4NO_3	1.125

The medium of Kinoshita,[2] often referred to as "medium K," is of the following composition:

	Grams in 1,000 Cc. of Water
$MgSO_4.7H_2O$	0 5
KH_2PO_4	1 0
NH_4NO_3	0 4

Katagiri and Kitahara used media containing 0.01 per cent $MgSO_4.7H_2O$, 0.1 per cent KH_2PO_4, 0.05 per cent $(NH_4)_2SO_4$ and 0.01 per cent $CaCl_2$ with 5 per cent of carbon-containing substance.

Kojic acid may be produced in the usual Czapek-Dox medium.

Ammonium nitrate is very satisfactory as a source of nitrogen Apparently better yields are obtained when the concentration of the salt is small, but sufficiently large to permit growth of the mold.

pH.—The optimum pH for the production of kojic acid under a given set of conditions must be determined by experimentation if this fact is not already known. The range of pH 2 to 5 or above has been used by various workers. Katagiri and Kitahara employed an initial pH of 5 0 to favor the growth of *A. oryzae* but found that a pH of 2.4 stimulated formation of kojic acid. A pH of 5.5 was found to be optimum for the fermentation of sucrose by Tamiya Barham and Smits obtained highest yields when using a pH range of 2 to 3.5 in the fermentation of xylose by *A. flavus* These men advanced the opinion that the optimum pH for the fermentation was the lowest one that the organism would tolerate (Compare with the citric acid fermentation.)

The addition of calcium carbonate to a kojic acid fermentation results in a greatly diminished yield of the acid. It is believed that the decreased yield is due to a change in the pH of the mash.

[1] MAY, O E, A J MOYER, P A WELLS, and H T HERRICK, *Jour Am Chem Soc*, **53**: 774 (1931)

[2] KINOSHITA, K, *Acta Phytochim (Japan)*, **3**: 31 (1927)

Effect of Added Substances.—In a survey of 40 organic compounds, May and his associates[1] found that ethylene chlorhydrin in a concentration of 100 mg. per liter produced a marked increase in the yield of kojic acid in a period of 10 days.

Oxalic, citric, formic, hydrochloric, and nitric acids inhibit the formation of kojic acid by fermentation.[2]

Temperature.—The temperature range of 29 to 35°C. is optimum for the fermentation. May and his associates advocated a temperature of 30 to 35°C. for *Aspergillus flavus* (a strain of the *A flavus-oryzae* group, secured from Dr. Thom as culture number 3538). A temperature of 29 to 31°C. was used by Kitahara and Katagiri; one of 35°C. by Barham and Smits, and one of 20°C. by Gould Sometimes the temperature may be dropped to 25°C. or lower after the fermentation has proceeded at a higher temperature for about 5 days, resulting in increased yields.

Duration of Fermentation.—The fermentation generally requires 9 to 20 days for completion, the period depending on the type of substrate, species of mold, the temperature, pH, and other factors. After the sugar has been consumed, the kojic acid may be utilized by the mold, resulting in decreased yields.

Yields.—Yields amounting to 50 to 60 per cent may be produced from glucose solutions by *A. flavus* Table 133 illustrates the nature of the yields obtained from various compounds by Katagiri and Kitahara

Other details concerning the fermentation may be ascertained by reference to some of the original papers cited at the end of this chapter.

Mechanism of Kojic Acid Formation.—There have been several schemes proposed to explain the production of kojic acid by fermentation. Some of these will be considered briefly.

Yabuta held that kojic acid arose directly from glucose by a simple process of oxidation and dehydration.
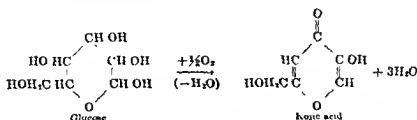
Kinoshita (1927) and Haworth (1928) offered a similar explanation for the origin of kojic acid. The basis for this theory appears to be that

[1] MAY, O E , G E WARD, and H T HERRICK. Centr Bakt Parasitenk , Abt II, 86 : 129 (1932)
[2] BARHAM, H N , and B L SMITS, Trans Kansas Acad Sci , 37 : 91 (1934)

TABLE 133.—AMOUNT OF KOJIC ACID PRODUCED BY *A. oryzae*[1]
(From 100 cc. of 20 days' culture containing 5 g. of substance)

Substance	Kojic acid		Observed by Sakaguchi (1032)	Observed by Tamiya (1932)
	Grams in 100 cc.	Yield on basis of sugar present, per cent		
Glycogen	0			
Inulin	0			0
Sucrose	0 65	14.8	..	
Maltose	1 45	34.8	+	+
Lactose	1.70	40.8	+	+
Trehalose.	±	±	..	+
Glucose.	0 095	2 3	+	0
Fructose	0 75	18 1	..	+
Mannose	0 40	10.2	+	+
Galactose	0 23	5 8	+	+
α-Methylglucoside	0 04	1.0	+	0
Rhamnose	0		+	0
Arabinose	0.08	0		0
Xylose	0.40	2.0	+	0
Dihydroxyacetone[2]	0.40	10.2	+	+
Glyceraldehyde	0 52	33.0	+	0
Methylglyoxal	±	±		
Inositol[3]	0	0		
Mannitol	0.01	0.9		+
Sorbitol[3]	±	±	+	
Dulcitol[3]	0 4	10 2		±
Erythritol	0 4	10 2		+
Glycerol	±	±	+	+
Na-glycerophosphate.	0 16	4 2	+	±
Ethylene glycol	0	0		0
Ethyl alcohol	0	0		
Methyl alcohol	0	0	+	
Gluconic acid[4]	0 34	9 4	±	0
Saccharic acid	0	0		0
Lactobionic acid	±	±		0
Arabonic acid	0	0		
Glyceric acid	0	0		
Succinic acid	0	0		±
Tartaric acid	0	0		0
Oxalic acid	0	0		
Acetic acid	0	0		
Pyruvic acid	0	0	±	0
Lactic acid	0	0		0
Glycolic acid	0	0		0

[1] KATAGIRI, H., and K. KITAHARA, *Mem. Coll. Agr., Kyoto Imp. Univ.*, No. 26 (Chem. Series 15) March, 1933

[2] The mixture of 2 per cent dihydroxyacetone and 3 per cent α-methylglucoside is used for the observation with dihydroxyacetone.

[3] Incubation for 14 days.

[4] Incubation for 40 days.

glucose yields relatively large quantities of the acid in comparison with some of the other carbon-containing materials. This theory does not explain how kojic acid is formed from compounds containing less than 6 carbon atoms, such as the pentoses, glycerol, and dihydroxyacetone, or from 7-carbon compounds.

Corbellini and Gregorini[1] (1930) advanced the suggestion that a pyrone nucleus is synthesized from 3-carbon compounds For example, 2 molecules of 3-carbon compounds may condense to form a molecule that by dehydration passes to kojic acid:

$$
\begin{matrix}
CH_2OH \\
| \\
CHOH \\
| \\
CHO
\end{matrix}
+
\begin{matrix}
CHO \\
| \\
CHOH \\
| \\
CHO
\end{matrix}
\rightarrow
\begin{matrix}
CH_2OH \\
| \\
CHOH \\
| \\
CHOH \\
| \\
CO \\
| \\
COH \\
| \\
CHOH
\end{matrix}
\xrightarrow{-2H_2O}
\text{Kojic acid}
$$
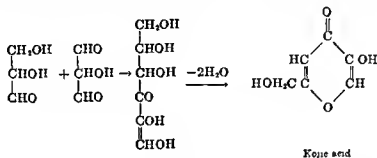
Pyrones are readily formed chemically from acyclic polyketones through dehydration

May and his associates share the view advanced by Corbellini and Gregorini that kojic acid is synthesized from some substance containing 2 or 3 carbon atoms They suggest that 1-hydroxyacetyl-3-formyl-3-hydroxyacetone may be the precursor of kojic acid, although the former compound has not been isolated from a kojic acid fermentation:

$$
\text{1-Hydroxyacetyl-3-formyl-3-hydroxyacetone} \xrightarrow{-H_2O} \text{kojic acid} + H_2O
$$
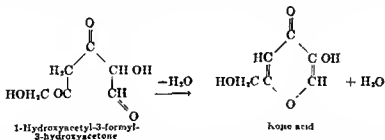
Birkinshaw, Charles, Lilly, and Raistrick[2] (1931) have proposed two theories to explain the mechanism of kojic acid formation. One theory suggests the formation of kojic acid through the condensation of acetaldehyde This theory is based upon the fact that ethyl alcohol is frequently found in mold fermentations, especially in those in which kojic acid is

[1] CORBELLINI, A, and B GREGORINI, Gazz chim ital, 60: 214 (1930)

[2] BIRKINSHAW, J H, J H V CHARLES, C H LILLY, and H RAISTRICK, Trans Roy Soc London, B220: 127 (1931)

one of the end products. The suggestion is also made that the presence of ethanol presupposes the occurrence of acetaldehyde as a precursor. Katagiri and Kitahara (1929) showed that acetaldehyde could be detected by sulphite fixation in several different types of media fermented by *Aspergillus oryzae* (Higuchi blue). Sakaguchi found that ethanol increased the yield of kojic acid from glucose solutions. He therefore believed that ethanol might be an intermediate in the fermentation.

The addition of fixing agents, such as sulphite and dimedon, to mashes being fermented by *A. tamarii* did not prevent kojic acid formation, nor could fixation products be isolated from the media, according to Gould.[1]

Katagiri and Kitahara (1933) have reported that no kojic acid could be detected in media that contained calcium hexosediphosphate or calcium and magnesium hexosemonophosphates. Furthermore, these investigators were unable to obtain kojic acid from media containing acetaldehyde; acetone; or pyruvic, parapyruvic, or acetoacetic acids

Thus, although acetaldehyde and ethanol are produced in some mold fermentations, there is no direct evidence to show that they are intermediate products in the formation of kojic acid.

The second theory proposed by Birkinshaw and his associates was that a reserve carbohydrate would be anabolized from the carbon-containing substance in the medium and that subsequently the reserve carbohydrate would be hydrolyzed to a compound that could be converted to kojic acid.

Research carried out by Gould has indicated that kojic acid is probably not produced from reserve carbohydrates. Gould grew mycelial mats of *A. tamarii* on media containing several different carbon-containing substances. The mats were washed free of kojic acid, dried, and then ground to a fine powder. The powder was substituted for sugar in the Czapek-Dox medium. No kojic acid was produced in 90 days.

Tamiya proposed that from the various constituents of the medium a hexose might be synthesized which would give rise to kojic acid.

In 1931, Challenger, Klein, and Walker reported that *A. oryzae* produced a yield of more than 30 per cent of the theoretical from dihydroxyacetone They proposed the following scheme

Dihydroxyacetone $\xrightarrow[+\frac{1}{2}O_2]{(-2H_2O)}$ kojic acid $+ 3H_2O$
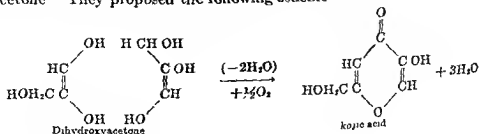
[1] GOULD, B S, *Biochem Jour*, **32**: 797 (1938)

Challenger and his associates thus assumed that dihydroxyacetone or glyceraldehyde were the logical intermediate products in the formation of kojic acid.

Katagiri and Kitahara (1933) concluded, as the result of extensive research, that dihydroxyacetone would be the most probable substance of those suggested to assume an important role in kojic acid formation.

References on Kojic Acid Fermentation

BARHAM, H N : Copper Precipitation Method for Kojic Acid Determination, *Ind Eng Chem (Anal Ed)*, **11**: 31 (1939)

———and B L SMITS: Kojic Acid—A Review, *Trans Kansas Acad Sci*, **37**: 91–113 (1934)

——— and ———: Production of Kojic Acid from Xylose by *Aspergillus flavus*, *Ind Eng Chem*, **28**: 567–570 (1936)

BERNHAUER, K : "Garungschemische Praktikum," 2d ed, Verlag Julius Springer, Berlin, 1939

BIRKINSHAW, J. H., J H V CHARLES, C. H LILLY, and H RAISTRICK: Studies in the Biochemistry of Micro-organisms. VII Kojic Acid (5-Hydroxy-2-hydroxy-methyl-γ-pyrone), *Trans Roy Soc London*, **B220**: 127–138 (1931).

CHALLENGER, F Some Fermentative Changes of Technical Interest, *Ind Chemist*, **6**: 97–100 (1930).

———, L KLEIN, and T. K. WALKER· The Production of Kojic Acid from Pentoses by *Aspergillus oryzae*, *Jour Chem Soc*, July, 1929, pp 1498–1505

———, ———, and ———· The Formation of Kojic Acid from Sugars by *Aspergillus oryzae*, *Jour. Chem Soc*, January, 1931, pp 16–23

CORBELLINI, A , e B GREGORINI. La formazione dell'acido kojico dal carbonio per azione dell' *Aspergillus flavus*

DI CAPUA · · · · · flavus

FRIEDEMA· · · · 31 (19

GOULD, B · · · ·ol *Aspergillus tamarii* Kita. Kojic Acid Production, *Biochem Jour*, **32**: 797 (1938)

HAWORTH, W N.· "Constitution of Sugars," Longmans, Green & Company, New York, 1929

KATAGIRI, H , and K KITAHARA The Formation of Kojic Acid by *Aspergillus oryzae*, *Bull Agr Chem Soc (Japan)*, **5**: 38–47 (1929)

——— and ——— The Formation of Kojic Acid by *Aspergillus Oryzae*, *Mem. Coll Agr , Kyoto Imp Univ*, No 26 1–29 (1933)

KINOSHITA, K : Über die Ernährung der Pilze mit den Kobaltammnkomplexsalzen, *Acta Phytochim (Japan)*, **3**: 31–50 (1927)

KLUYVER, A J , and L H C PERQUIN· Über die Bedingungen der Kojisäurebildung durch *Aspergillus flavus* Lnk, *Biochem Zeit*, **266**: 82–85 (1933)

MAURER, K Die Überführung von einfachen Zuckern in γ-Pyron-Derivate und die Darstellung weiterer ungesättigter Anhydro-zucker, III, *Ber*, **63B**: 25–34 (1931)

MAY, O E , A J MOYER, P A WELLS, and H. T. HERRICK The Production of Kojic Acid by *Aspergillus flavus*, *Jour Am Chem Soc*, **53**: 774–782 (1931)

———, G. E. WARD, and H. T. HERRICK: The Effect of Organic Stimulants upon the Production of Kojic Acid by *Aspergillus flavus*, *Centr. Bakt. Parasitenk.*, Abt II, **86**: 129–131 (1932).

SAITO, K.: Über Die Säurebildung Bei *Aspergillus Oryzae* (Vorläufige, Mitteilung), *Botan. Mag. Tokyo*, **21**: 240 (1907).

SAKAGUCHI, K : *Jour. Agr. Chem. Soc. (Japan)*, **8**: 265 (1932).

TAMIYA, H.: Studien über die Stoffwechselphysiologie von *Aspergillus niger*, I, *Acta Phytochim. (Japan)*, **3**: 51–173 (1927).

———: Über die Verwendbarkeit von verschiedenen Kohlenstoffverbindungen im Bau- und Betriebstoffwechsel der Schimmelpilze, *Acta Phytochim. (Japan)*, **5**: 1–129 (1932).

——— und T. HIDA: Vergleichende Studien über die Säurebildung, die Atmung, die Oxydasereaktion und das Dehydrierungsvermögen von Aspergillusarten, *Acta Phytochim. (Japan)*, **4**: 343 (1929).

TRAETTA-MOSCA, F.: La fermentazione di alcuni zuccheri, mediante l'*aspergillus glaucus*, con alcune considerazioni sulla fermentazioni alcoolica, *Ann. chim applicata*, **1**: 477–492 (1914).

———e M. PRETI: Azione dell' *Aspergillus Glaucus* sulla glicerina, *Gazz. chim ital*, **51**: (II): 269–277 (1921).

YABUTA, T : On Koji Acid, a new organic acid formed by *Aspergillus oryzae*, *Jour. Coll. Agr., Tokyo Imp Univ.* **5**: 51–58 (1912).

———: Original Communication of the Eighth International Congress of Applied Chemistry (Appendix), **25**: 455–462 (1912).

———: The Constitution of Kojic Acid, a γ-Pyrone Derivative Formed by *Aspergillus Oryzae* from Carbohydrates, *Jour. Chem Soc. (Japan)*, **125**: 575–587 (1924).

WIKMAN, N.: *Zeit. physiol. Chem.*, **132**: 104–108 (1924).

CHAPTER XXXI

THE PRODUCTION OF LACTIC ACID BY MOLDS

Recent investigations have indicated that the production of d-lactic acid by molds has commercial possibilities and certain apparent advantages.

Historical.—In 1894 the suggestion was made by Eijkmann that the acid formed by *Mucor rouxii* was lactic acid. Chrząszcz confirmed Eijkmann's suggestion in 1901 [1]

Patents were issued to Boullanger (1899) for the production of lactic acid by a mold described as "Lactomyces," an organism now believed to be a species of *Rhizopus*

Saito reported the production of small quantities of l-lactic acid from glucose solutions by *R chinensis* in 1911.

According to Ehrlich (1919), small quantities of d-lactic acid, l-malic acid, and succinic acid, but principally fumaric acid, are produced by some species of *Rhizopus*

Takahashi and his associates (1925) demonstrated that as much as 38 per cent of the glucose fermented was converted to l-lactic acid by certain species of *Rhizopus* in some of their experiments Variable quantities of acetic, formic, fumaric, l-malic, and succinic acids and ethyl alcohol were produced at the same time. In later work, Takahashi and Asai (1933) showed that four species of *Mucor* produced traces of lactic acid from glucose media Traces of pyruvic and succinic acids were likewise found, but ethyl alcohol was the principal product formed

Miyaji (1930) reported the production of d-lactic and succinic acids from glucose media by a new species of *Monilia*, isolated from a commercial culture of soy sauce.

In 1931, results of work carried out by Kanel, in which he used a species of *Rhizopus* similar to *R japonicus*, were published Yields of as much as 38 to 40 per cent of lactic acid, on the basis of the carbohydrate consumed, were obtained from invert sugar and from starch The nutrient medium contained 10 per cent sugar and 4 per cent calcium carbonate Fumaric acid was occasionally recovered from the older cultures, the quantity formed being a function of the conditions of culture and other factors.

[1] Chrząszcz, T. *Centr Bakt Parasitenk*, Abt II, 7: 326 (1901)

Ward and his associates,[1] in studying the biochemical activities of fungi of the genus *Rhizopus*, found several species that converted glucose to d-lactic acid in the presence of calcium carbonate Two strains, *R. oryzae* 394 and *R. oryzae* Went and Geerligs 395, demonstrated particularly good results, yields up to 62 per cent, or greater, being obtained by a surface-culture method.

Methods of Production.—In general, d-lactic acid may be produced by selected strains of molds by a surface-culture method or by a rotary-fermenter method. The research of Ward, Lockwood, Tabenkin, and Wells indicates the superiority of the rotary-fermenter process over the surface-culture process.

The following descriptions are based on the research carried out by scientists of the *Industrial Farm Products Research Division, Bureau of Chemistry and Soils, U.S. Department of Agriculture.*

Surface-culture Method.—In this method, the molds are grown in Pyrex Erlenmeyer flasks, each of which contains 75 cc. of nutrient glucose medium. Sterilized calcium carbonate is added to the flasks at the rate of 4 or 5 g. per flask at the time of inoculation, for in the absence of calcium carbonate only a small amount of acid is formed.

The Mold.—Lactic acid has been produced from the following molds: *Rhizopus orrhizus*, *R. chinensis*, *R. pseudochinensis*, *R. elegans*, *R. oryzae*, *R. salebrosus*, *R. shanghaiensis*, *R. stolonifer*, *R. tritici*, certain Mucors and at least one Monilia. Of the foregoing molds, *R. oryzae* Went and Geerligs 395 and *R. oryzae* 394 are outstanding in their ability to produce d-lactic acid. The authors have found no records indicating the production of lactic acid by aspergilli, penicillia, or fusaria.

Preparation of the Mold Suspension—Lockwood, Ward, and May[2] prepared their mold suspension in the following manner: The mold was grown on sterile moist bread for at least 4 days at 30°C. in order to produce an abundance of spores. Stolons and sporangiospores were placed in a tube or bottle containing sterile water and shaken vigorously to liberate the spores. Using aseptic precautions, the stolon mass was then removed An estimate of the spore content of the suspension was made next, and the size of the inoculum regulated to provide 12 5 to 100 million spores per 200-cc. Pyrex Erlenmeyer flask containing 75 cc. of nutrient glucose medium.

The Medium—The composition of the medium used for the production of lactic acid was as shown in the table on page 656.

[1] WARD, G. E., L B. LOCKWOOD, O. E. MAY, and H T. HERRICK, *Jour. Am. Chem. Soc*, **58:** 1286 (1936)

[2] LOCKWOOD, L B., G. E. WARD, and O. E. MAY, *Jour. Agr. Research*, **53:** 849 (1936)

TABLE 131—The Effect of Different Sources of Nitrogen on the Metabolism of R. oryzæ.[a]

Nitrogen source	Quantity per liter, grams	Weight of mycelium, grams	Glucose consumed, grams	Calcium dissolved			d-Lactic acid		Fumaric acid	
				Total, grams	Due to d-lactic acid, per cent	Due to fumaric acid, per cent	Weight, grams	Weight, yield,[1] per cent	Weight, grams	Weight, yield,[1] per cent
NaNO₃	3 00	0 000	0 0	0 00	0	0	0 00	0	0 000	0 0
NaNO₂	2 56	0 026					4 03	49 8	0 009	1 2
NH₄NO₃	2 58	0 596	8 1	1 05	86 0	3 2	3 30	54 2	0 082	1 3
(NH₄)₂SO₄	2 30	0 301	6 2	0 79	95 0	3 5	4 13	57 4		
Cl	1 98	0 370	7 2	1 05	87 5		5 44	63 3	0 217	2 5
	2 14	0 613	8 6	1 24	87 5	6 0	4 87	58 7	0 312	3 8
urine	3 30	0 350	8 3	1 26	86 0	8 6	4 05	57 0	0 200	2 9
...tamic acid	5 46	0 350	7 1	1 08	86 0	0 0	3 37	57 1	0 183	3 1
...rine	2 67	0 257	5 9	0 87	83 0	7 2	4 95	59 0	0 189	2 3
Peptone	3 24	0 219	8 3	1 29	83 0	5 0				

[a] 175 cc. of 15.1 per cent glucose nutrient solution contained 0.25 g MgSO₄.7H₂O and 0.3 g KH₂PO₄ per liter; 5 g CaCO₃ per flask. Duration, 14 days. Temperature, 30°C.

Lockwood, L. B., O. E. May, and O. E. Ward, Jour. Agr. Research, 53: 849 (1936).

[1] Grams of acid produced divided by grams of glucose consumed.

Glucose (commercial grade)	15 per cent
KH₂PO₄..	0 3 g. per liter
NH₄NO₃, (NH₄)₂SO₄, or urea, to yield......	0.5 g nitrogen per liter
MgSO₄·7H₂O	0.25 g. per liter

Effect of Variations in the Constituents of the Medium.—Lockwood, Ward, and May studied the physiology of *R. oryzae* Went and Geerligs[1] and brought out many important facts. The optimum concentration of glucose was 15 per cent, determined on the basis of lactic acid formed, the glucose consumed, and the weight of the mycelia.

The best yields of lactic acid were produced when the concentration of KH₂PO₄ was 0.6 g. per liter. Doubling this amount of the salt did not increase the yield of lactic acid.

Ammonium chloride; ammonium nitrate; ammonium sulphate; d-l-alanine; d-glutamic acid; glycine; peptone; and urea served as satisfactory sources of nitrogen. Sodium nitrate failed to produce growth, while sodium nitrite produced only a slight amount of growth. Table 134 shows the effect of different sources of nitrogen on the metabolism of *R. oryzae*.

Effect of Zinc Sulphate.—When calcium carbonate was a constituent of the culture medium, the addition of 10 mg. of zinc, in the form of zinc sulphate, caused an increase in the weight of mycelium, in the glucose consumption, and in the absolute quantity of lactic acid formed by *R. oryzae* Went and Geerligs 395. The yield of d-lactic acid, calculated in terms of grams of acid formed divided by grams of glucose utilized, became progressively smaller as the concentration of zinc was increased, however. In the absence of calcium carbonate, there was no increase in the glucose consumption.

Effect of Other Salts.—Ferric and chromium ions, both in the presence and absence of calcium carbonate, produced no apparent effect on glucose consumption or lactic acid production.

Temperature.—A temperature of 30°C. is favorable for the production of lactic acid. At 40°C., the growth of the mold is more rapid but the yield of acid is less than at 30°C.

Incubation Period.—Cultures are usually incubated for 16 to 21 days. The effect of the incubation period on results obtained with *R. oryzae*[2] is shown in Table 135.

Fumaric Acid—Production of fumaric acid by *R. oryzae* Went and Geerligs 395 is a variable factor. For example, Lockwood, Ward, and May found that a temperature of 40°C. is more favorable for its produc-

[1] *Ibid.*
[2] LOCKWOOD, L. B., G. E. WARD, and O E. MAY, *Jour. Agr. Research*, **53**: 849 (1936).

tion than 30°C.; that ammonium nitrate (NH_4NO_3) in a concentration greater than 6 g. per liter and that high concentrations of glucose inhibit its formation; and that it is not produced during the early part of the fermentation but only after several days.

TABLE 135 —THE EFFECT OF THE LENGTH OF THE INCUBATION PERIOD ON THE METABOLISM OF *R. oryzae*[1]

Age, days	Weight of mycelium, grams	Glucose consumed, grams	Calcium dissolved			d Lactic acid		Fumaric acid	
			Total, grams	Due to d-lactic acid, per cent	Due to fumaric acid, per cent	Weight, grams	Weight yield,[2] per cent	Weight, grams	Weight yield,[2] per cent
5	0.040	1 3	0 067	..	..	0 060	..	0	0
9	0 039	2 0	0 24	87	0	0 93	46.5	0	0
13	0 220	6 3	0 85	88	0	3 36	53 3	0	0
17	0 839	10 5	1 28	78	8 7	4 50	42 9	0 314	3 0
21	1 032	11 2	1 44	72	13 0	4 66	41 6	0 664	5.0

[1] 75 cc of 15 per cent glucose, 0 25 g MgSO₄ 7H₂O, 0 3 g KH₂PO₄, and 2 88 g. NH₄NO₃ per liter, 5 g CaCO₃ per flask Temperature 30°C
[2] Grams of acid produced divided by grams of glucose consumed

Other End Products.—Acetic, l-malic, and succinic acids and ethyl alcohol have been detected in traces, or in small quantities, in some glucose media fermented by *R. oryzae*

Yields.—Yields of as high as 62 to 67 per cent lactic acid have been obtained by the surface-culture method on the basis of the sugar consumed.

The largest quantity of *d*-lactic acid is produced just before sporulation of the mold.

The Rotary-fermenter Process.—Lactic acid is produced in 30 to 35 hr [1] in rotating aluminum drums of the type described in the chapter on the gluconic acid fermentation. This process is superior to the surface-culture process both from the standpoint of the shorter fermentation period and of the quantity of acid produced from the sugar consumed, yields of 70 to 75 per cent of acid being common

Procedure.—Spores are produced for inoculation purposes by growing the mold on sterile moist bread A special germination medium is inoculated to contain 420 million spores per 1.5 liters of the medium. The germination medium containing the spores is shaken for 24 hr. at 30°C. in a 4-liter glass bottle equipped with an outlet tube. By using this medium, the composition of which is shown in Table 136, a saving in fermentation time is effected

[1] WARD, G E , L B LOCKWOOD, B TABENKIN, and P A. WELLS, *Ind. Eng Chem*, **30**: 1233 (1938)

A 3-liter portion of the fermentation medium is inoculated with 250 cc of the germinated spore culture and placed in the aluminum drum. The drum is operated at a gauge pressure of 5 lb. per sq. in. and a rotation speed of 13 r.p.m. Air, measured at the exit, flows through at the rate of 150 cc. per min., while the temperature is maintained at 35°C.

TABLE 136 —SOME MEDIA USED IN LACTIC ACID PRODUCTION[1]

Substance	Germination medium, grams	Fermentation medium, grams
Glucose (91.5 per cent, commercial)	110	150
Urea ...	2.0	2.0
KH₂PO₄	0.60	0 60
MgSO₄ 7H₂O	0.25	0 25
ZnSO₄ 7H₂O	0 088	0.044
CaCO₃	10 0	*
Octadecyl alcohol	. . .	0 03†
Distilled water	to 1 liter	to 1 liter

[1] WARD, G. E., L. B LOCKWOOD B TABENKIN, and P. A. WELLS, *Ind Eng Chem,* 30: 1233 (1938)

* 200 g to each 3-liter portion—sterilized separately

† Dissolved in 1 7 cc ethanol (added to prevent excessive foaming of the fermentation medium during the rotation of the drum).

On account of the limited solubility of calcium lactate and in view of the higher yields obtained by this method, it is necessary to use a somewhat lower concentration of glucose than is the case in the surface-culture method When a precipitate of calcium lactate settles out, forming a white mass, fermentation is much inhibited If the settling occurs before all the sugar is utilized, it is particularly objectionable. By using 13 per cent, or less, glucose, satisfactory results are usually obtained.

Results of a Typical Experiment.—The following data are quoted to illustrate the result of a typical fermentation carried out by the rotating-drum method, using *R. oryzae* Went and Geerligs:

	Grams per 100 Cc [1]
Original glucose concentration	13 3
Glucose consumed	12 8
Ethyl alcohol produced	0 62
Calcium in solution	2 245
Lactic acid equivalent to dissolved calcium	10 12
Lactic acid found by analysis	9 66
Acidity due to lactic acid, per cent	95 4
Yield of d-lactic acid, based on glucose consumed, per cent	75 5

[1] WARD G E., L B LOCKWOOD, B TABENKIN, and P A WELLS, *Ind Eng Chem,* 30: 1233 (1938)

The rate at which glucose was consumed in this experiment is indicated in Fig. 89 on this page.[1] It will be observed that there was but little consumption of glucose during the first 15 hr., but during the rest of the experiment the sugar was consumed at a fairly rapid rate.
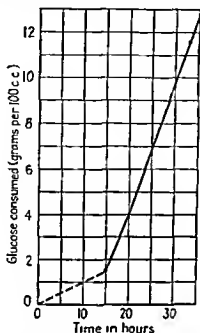
Fig. 89.—Course of a typical *d*-lactic acid submerged fermentation induced by *Rhizopus oryzae* [*Courtesy of Ward, Lockwood, Tabenkin, and Wells, Ind. Eng. Chem.*, **30**, 1233 (1938)]

Respiration and Lactic Acid Production.—Waksman and Foster[2] have carried out research concerning respiration and lactic acid production by a species of the genus *Rhizopus*. They have suggested reactions to explain the mechanism of these important processes.

Advantages of Mold Process.—Although *d*-lactic acid, a physiologically important acid (known as sarcolactic acid, also) is not produced commercially by molds at present, this process has, as pointed out by Ward and his associates, several apparent advantages. Owing to the fact that urea is used as the source of nitrogen, a lactic acid free from color and of relatively high purity may be produced. The fermentation is rapid, and salts of lactic acid are easily recovered. Furthermore, this process provides a source of *d*-lactic acid, which may be used for the preparation of crystallized *d*-lactic acid.

[1] WARD, LOCKWOOD, TABENKIN, and WELLS, *loc. cit.*

[2] WAKSMAN, S. A., and J. W. FOSTER, *Jour. Agr. Research*, **57**: 873 (1938)

References on Production of Lactic Acid by Molds

Chrzaszcz, T.: Die "Chinesiche Hefe," *Mucor cambodja*, eine neue technische Pilzart, nebst einigen Beobachtungen über *Mucor rouxii*, Centr. Bakt. Parasitenk., Abt. II, 7: 326 (1901).

Ehrlich, F.: Über Fumarsäure—Gärung des Zuckers, *Ber.*, 52: 63 (1919).

Eijkmann, C.: Mikrobiologisches Untersuchungen über die Arraklsfabrikation in Batavia, Centr. Bakt. Parasitenk., Abt. I, 16: 97 (1894).

Kanel, E.: Lactic Acid Formation in the Culture of a Fungus of the *Rhizopus* Species, *Microbiology U.S.S.R.*, 3: 259 (1934).

————: Comparative Study on the Mechanism of the Respiratory Process of Two Closely Related Strains of *Rhizopus nigricans* in Connection with the Differentiation of the Thallome and the Changes in External Conditions. *Microbiology U.S.S.R.*, 4: 636 (1935).

Lockwood, L. B., G. E. Ward, and O. E. May: The Physiology of *Rhizopus oryzae*, *Jour. Agr. Research*, 53: 849 (1936).

———— and A J. Moyer: The Production of Chemicals by Filamentous Fungi, *Bot. Rev.*, 4: 140 (1938).

Miyazi, K.: Notiz über "*Monilia tamari*" Kagami Kenkyu Hokoku, *Research Bull Gifu Imp. Coll. Agr.*, 10: 1–5 (1930).

Saito, E.: Ein Beispiel von Milchsäurebildung durch Schimmelpilze, Centr. Bakt. Parasitenk., Abt. II, 29: 289 (1911).

Takahashi, T., and T. Asai: On the Products of Fermentation by *Mucor* Group II The Products of Fermentation in Presence of Ca-Carbonate, Centr. Bakt. Parasitenk., Abt. II, 89: 81: 1933.

———— and K. Sakaguchi: Studies on the Acids Formed by *Rhizopus* Species, *Jour. Agr. Chem. Soc. (Japan)*. 1: 40 (1925).

————, and T. Asai: Studies on the Acids Formed by *Rhizopus* Species II Formation of Ethyl Alcohol from Tartaric or Fumaric Acid, *Bull. Agr. Chem. Soc. (Japan)*, 2(5): 61 (1926).

Waksman, S A., and J. W. Foster: Respiration and lactic acid production by a fungus of the genus *Rhizopus*, *Jour. Agr. Research*, 57: 873–900 (1938).

———— and I. J. Hutchinson: Lactic Acid Production by Species of *Rhizopus*, *Jour. Am Chem Soc*, 59: 545–547 (1937).

Ward, G E., L. B Lockwood, O. E. May, and H. T. Herrick: Studies in the Genus *Rhizopus*. I. The Production of Dextro-lactic Acid, *Jour. Am. Chem. Soc.*, 58: 1286 (1936).

————, B. Tabenkin, and P. A. Wells: Rapid Fermentation Process for Dextro-lactic Acid, *Ind. Eng. Chem.*, 30: 1233 (1938).

Some Patents

Boullanger, E.: Improvements in the Manufacture of Lactic Acid, British Patent 13,439, 1899, also German Patent 118063, 1901.

Ward, G. E., L B Lockwood, and O. E. May: Fermentation Process for the Manufacture of Dextro-lactic Acid, U.S Patent 2,132,712, Oct. 11, 1938.

CHAPTER XXXII

MANNITOL PRODUCTION BY MOLDS

Mannitol (CH_2OH ($CHOH$)$_4$ CH_2OH), an alcohol, occurs naturally in manna, an exudate from certain plants, prominent among which is the manna ash tree, *Fraxinus ornus* It is formed as a fermentation product from fructose by certain bacteria.

Braconnot (1811) and Vauquelin (1813) reported that mannitol was found to be a constituent of the tissue of some of the higher fungi. Mannitol has since been found in the mycelium of some of the lower fungi as well. As such, it is regarded as a reserve food product rather than as a product of fermentation

Several fungi, however, produce mannitol as a fermentation product from nutrient sugar solutions. Mannitol is produced from glucose in a Czapek-Dox solution by certain white aspergilli,[1] by *Aspergillus elegans*, by *A. nidulans*, by *Helminthosporium geniculatum*, by a species of *Clasterosporium*, by *Byssochlamys fulva*, and by *Penicillium chrysogenum* It is produced from sucrose, together with itaconic acid, by *A. itaconicus*; and from glycerol by molds of the *A glaucus* group Ethyl alcohol is formed by two of the foregoing fungi, namely, *H geniculatum* and the species of *Clasterosporium*, as one of the main products.[2] Other products are formed in small quantities by some of these fungi, such as glycerol, acetaldehyde, succinic acid, and malic acid.

Pruess and his associates[3] have isolated mannitol from *A. fischeri* and *A. oryzae*.

Yields of as much as 50 per cent mannitol, based on the sugar utilized, have been produced by a white species of *Aspergillus* (Thom 4640 489) from the Czapek-Dox solution of glucose (2 g of $NaNO_3$, 1 g of KH_2PO_4; 0.5 g of KCl; 0 5 g. of $MgSO_4$ 7 H_2O; 0 01 g $FeSO_4$ 7 H_2O; 50 g. of glucose and water to 1,000 cc)

Two other species of white aspergilli, a strain of *A. elegans* and five

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[1] BIRKINSHAW, J H., J. H V CHARLES, A C HETHERINGTON, and H RAISTRICK, *Trans Roy Soc London*, 220B[1] 153 (1931)

[2] BIRKINSHAW, J H , and H. RAISTRICK, *Trans Roy Soc London*, 220B: 331 (1931)

[3] PRUESS, L M , W H. PETERSON, and E B FRED, *Jour. Biol. Chem*, 97: 483 (1932)

strains of *A. nidulans*, were investigated by Birkinshaw and his associates.[1] The metabolic solutions were incubated at 23 or 24°C. usually, for periods of 2 to 10 weeks. Control of aeration was found to be important. Higher yields were obtained by restricting the air supply, this being effected by keeping the flasks closed to all air supply except the small amounts of sterilized air that were passed through the flasks during a 30-min. period once a day. Unrestricted aeration resulted in lower yields, for the "water-soluble product" formed was used up as the available glucose disappeared. Some of the highest yields were obtained when the exhausted metabolism solutions were replaced with fresh nutrient glucose solutions. As a result of using the mycelia over again, shorter fermentation periods were required.

The same strain of *Aspergillus*[2] produced yields of about 35 per cent *d*-mannitol from glucose, mannose, and galactose on the basis of the sugar utilized. A smaller yield of *d*-mannitol was obtained from xylose, a pentose. The yield from arabinose was poorer than that from xylose. No mannitol was produced from fructose, which is readily fermented by some bacteria and reduced by chemical means to *d*-mannitol.

Byssochlamys fulva, Olliver and Smith,[3] an ascomycete and a cause of spoilage in processed fruits, produces mannitol from glucose to the extent of about 30 per cent, on the basis of the sugar consumed, when grown in the Czapek-Dox solution. The inoculated solutions were incubated at 24°C. Byssochlamic acid ($C_{18}H_{20}O_6$; m.p. 163.5°C) was obtained in yields of about 0.5 per cent along with mannitol.

The following equation represents the formation of *d*-mannitol from *d*-glucose:
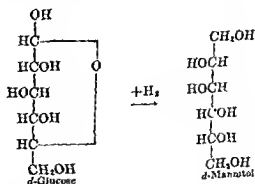
The mechanism by which *d*-mannitol is produced from the pentoses is not known at present.

[1] Birkinshaw, Charles, Hetherington, and Raistrick, *loc cit*
[2] Coyne, F. P., and H. Raistrick, *Biochem Jour.*, 25: 1513 (1931)
[3] Raistrick, H., and G Smith, *Biochem. Jour*, 27: 1814 (1933)

References on Mannitol Production

BIRKINSHAW, J. H.: Biochemistry of the Lower Fungi, *Biol. Rev*, **12**: 357 (1937).

——, J. H. V. CHARLES, A. C. HETHERINGTON, and H. RAISTRICK: Studies in the Biochemistry of Micro-organisms   IX   On the Production of Mannitol from Glucose by Species of *Aspergillus*, *Trans Roy Soc London*, **220B**. 153 (1931).

—— and H. RAISTRICK: Studies in the Biochemistry of Micro-organisms   XVII The Products of Glucose Metabolism Formed by Various Species of Fungi *Trans Roy Soc. London*, **220B**: 331 (1931).

BRACONNOT, H.: Sur la nature des champignons, *Ann chim*, **79**: 265 (1811).

——: Nouvelles recherches analytiques sur les champignons pour servir de suite à celles qui ont été insérées dans les tons, *Ann chim*, **87**: 237 (1813).

CLUTTERBUCK, P. W . Recent Developments in the Biochemistry of Moulds, *Jour Soc Chem Ind*, **55**: 55T (1936).

COYNE, F. P, and H RAISTRICK: Studies in the Biochemistry of Micro-organisms XX   On the Production of Mannitol from Hexoses and Pentoses by a White Species of *Aspergillus*, *Biochem. Jour*, **25**: 1513 (1931).

KINOSHITA, K . Über die produktion von Itaconsäure und Mannit durch einen neuen Schimmelpilz, *Aspergillus itaconicus*, *Acta Phytochim (Japan)*, **5**: 271 (1932).

PRUESS, L M', W. H PETERSON, and E B FRED Isolation and Identification of Ergosterol and Mannitol from *Aspergillus fischeri*, *Jour. Biol. Chem.*, **97**: 483 (1932).

RAISTRICK, H , and G SMITH' Studies in the Biochemistry of Micro-organisms. XXXV   The Metabolic Products of *Byssochlamys Fulva* Olliver and Smith, *Biochem Jour*, **27**: 1814 (1933).

—— and W YOUNG· Studies in the Biochemistry of Micro-organisms   X. The Estimation of Mannitol in Fermentation Solutions, *Trans Roy Soc London*, **220B**. 173 (1931).

VAUQUELIN. Expériences sur les champignons, *Ann chim*, **85**: 1 (1813).

YAMASAKI, I , und M SHIMOMURA Über die *d*-Mannitbildung aus Glycerin durch Schimmelpilze der *Aspergillus-glaucus*-Gruppe, *Biochem Zeit*, **291**: 340 (1937).

MOLD ENZYME PREPARATIONS: USES AND PRODUCTS

Reference has been made in Chap. XXIV to the types of enzymes that are elaborated by molds, while the use of living molds and mold preparations to saccharify starch in the manufacture of industrial alcohol has been discussed in Chap. III. Several enzyme preparations will now be considered in greater detail.

Commercial Amylase.—Commercial amylase or diastase is marketed under a variety of trade names, for example, Taka-diastase, Kashiwagi-diastase, Digestin, Polyzyme, Protozyme, and Oryzyme. Such preparations are generally mixtures of various enzymes Purified enzyme preparations may be secured, however.[1]

Preparation.—The mold that is to be used for the enzyme preparation may be grown in trays in shallow layers or in rotating drums.

In the former method, which is the older one, bran (wheat or rice) is moistened thoroughly, steamed for 1 to 2 hr. to make soluble the starch and to destroy the undesirable microorganisms present, and then cooled to 25 to 30°C.[2] This cooled material is inoculated with the spores of a selected strain of *Aspergillus oryzae*. The inoculated and well-mixed mass is spread in trays, preferably with false bottoms, or on a suitable base, to a depth of approximately 1.5 in.[3] The temperature is maintained close to the optimum for the growth of the mold, usually about 30°C. A humidity sufficiently high to prevent drying of the bran and ventilation adequate to supply sufficient oxygen and to carry away some of the carbon dioxide are essential. The mold develops rapidly, and in 40 to 48 hr a maximum of desirable enzymes are available in the mold mass. The product may be dried to prevent bacterial action; or the mass may be extracted at once with water (1 to 2 volumes for each volume of product), filtered or strained, and preserved in a manner suitable for the purpose for which the preparation is to be used.

If the extract is to be used for food or for medicinal purposes, sufficient ethanol is added to produce 70 per cent saturation. The enzymes are

[1] KITANO, T , *Jour Soc Chem. Ind. (Japan)*, 40: 37 (1937).
[2] WAKSMAN, S A., and W C. DAVISON, "Enzymes," The Williams & Wilkins Company, Baltimore, 1926
[3] TAKAMINE, J , *Jour. Ind. Eng. Chem.*, 6: 824 (1914)

precipitated by the alcohol. The precipitate is washed, and thus dehydrated, with strong ethanol, dried and finally powdered. The resultant powder is whitish to whitish-yellow in appearance.

An alternate method is to concentrate the extract to a heavy sirup, using vacuum and a temperature of 30 to 40°C.

In case the enzyme preparation is not to be used as a food, the aqueous extract may be preserved by the use of a chemical antiseptic. The addition of sodium chloride to a concentration of about the saturation point (20 per cent) has been recommended.[1] Thymol, tricresols, phenol, and other substances have been used as preservatives.

According to Harada,[1] the essential factors in the preparation of enzymes are (1) the quality of the bran; (2) the moisture content of the bran; (3) the pH, the temperature, and the time of incubation; (4) the humidity; and (5) the use of sterilization. The selection of an appropriate mold is, of course, of prime importance[2] as not all strains of the *A. flavus-oryzae* group are equally potent in enzyme production.

The production of amylase preparations by the use of rotating drums with controlled aeration and temperature has been described by Underkofler, Fulmer, and Schoene[3] (see Chap. III). Products prepared by this method were high in potency.

Continuous Tray Method for Producing Mold Enzymes.—Jeffreys[4] has described a method for producing mold enzymes by a continuous tray method. He states that it eliminates considerable of the labor normally required for handling trays in older processes. The method consists of preparing an inoculum, sterilizing the substrate and cooling it, inoculating the substrate, placing the inoculated substrate in trays, loading the trays on trucks, incubating the trays in special incubators, drying the mold product in tunnels, and grinding the dried product and bagging it. A diagram of the process is shown in Fig. 90.

Preparation of the Inoculum.—The mold *A. oryzae* is carried on suitable agar slants These cultures are used to inoculate a sterile moist bran medium in 2-qt. jars. After adequate sporulation has taken place, the contents of a jar may be used to inoculate 100 lb. of sterilized and cooled bran or mother culture The latter is grown in specially designed shallow trays (Fig 91) equipped with perforated covers A piece of newspaper is placed over the top of the tray before the cover is put on, which, according to Jeffreys, prevents contamination and absorbs moisture. The trays

[1] Harada, T, *Ind Eng Chem*, 23: 1421 (1931).
[2] Oshima, K, and M B Church, *Jour Ind Eng Chem*, 15: 67 (1923).
[3] Underkofler, L A, E. I. Fulmer, and L Schoene, *Ind Eng Chem*, 31: 734 (1939)
[4] Jeffreys, G A, *Food Inds*, 20: 688 (1948)

Fig. 90.—Diagram of process for producing mold enzymes by tray method. [Courtesy of G. A. Jeffreys, Food Inds., **20:** 688 (1948).]

containing the mother cultures are placed on racks in special incubator rooms in which the temperature is maintained at approximately 78°F. by means of forced-draft circulation and heating and cooling coils. After a suitable incubation period, the mold-spore preparation is ready for drying or for inoculation purposes. The tray covers are removed to facilitate drying of the mother cultures.

Preparation and Inoculation of Substrate. The substrate is fed onto a conveyor by a percentage feeder, where it is mixed with required amount
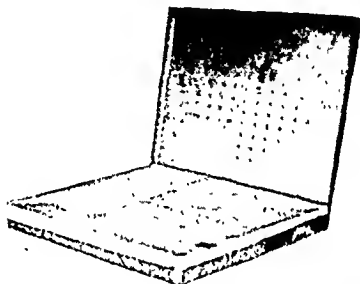
Fig. 91. Culture tray. [Courtesy of O. A. Jeffreys, Food Inds., 20: 688 (1948).]

of water. The mixture is heated to 190°F., or higher, with steam and maintained at this temperature for approximately 15 min. The steamed mixture is dropped onto a conveyor where cooling is brought about by a combination of cold, filtered air and a water jacket.

Moist and dried spores are used to inoculate the prepared substrate, the inoculum amounting to about 0.5 per cent on a dry basis. The moist spores are prepared by transferring the undried mother culture to a perforated basket in a tank and suspending the spores in sterile water, using mechanical agitation. The spore suspension is forced to a blending mixer by means of a proportioning pump where it is sprayed upon the substrate. At the same time, the substrate is inoculated with dry spores which are fed in from a bin by means of a percentage feeder and a blower. The substrate and spores are then thoroughly mixed together.
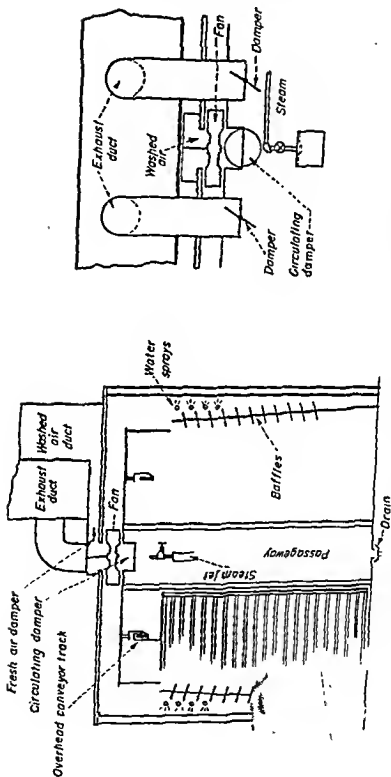
Fig. 92.—Culturing tunnel. [Courtesy of G. A. Jeffreys, Food Inds., 20: 688 (1948).]

Traying and Incubation —The inoculated substrate is dropped onto a spreader which distributes it uniformly in sterilized trays. The loaded trays are conveyed to a loading point and loaded onto a truck, the latter holding 20 trays, 4 in. apart. The trucks are pushed into culturing tunnels (special incubators) on overhead trolleys. Air is circulated in the culturing tunnels by means of fans located 6 ft. apart between the ceiling and subceiling which draw in the air, fresh or recirculated. (Refer to Fig. 92.)

During incubation the temperature of the substrate is kept from rising above 37°F by circulating cool humidified air through the culturing tunnels. When it becomes necessary to warm the air of the tunnels it is accomplished by the use of thermostatically controlled steam jets.

Drying and Bagging —After growth on the substrate has reached an optimum, usually after 24 to 30 hr., the trucks with their contents are transported into the drying tunnels which consist of primary and secondary stages with a center entrance. The air flows parallel to the direction of the movement of the trucks in the first stage and countercurrent to their direction in the secondary stage. The temperature of the enzyme-containing product is not allowed to exceed 110°F during the drying process, where in the first stage content is reduced to about 8 per cent.

The dried material is given that I bagged or it may be extracted.

Harvest of Trays —After the content of the trays are removed they are carried on a chain belt in the inverted position to conveyors which cleans them. They are next conveyed through screens where they are subjected to a temperature of 200°F for 3 min. After passing out, and cooling, they are ready for reloading.

In one method of making *koji*,[1] whole brown rice is used. The rice is cleaned, soaked for a few hours in water, and then steamed. The grains are thus swelled and cracked or burst. The steamed rice is spread out in trays in a layer approximately ½ in. thick to cool. Spores of *A. oryzae* are mixed thoroughly with the rice, which may then be heaped and covered with damp cloths to favor the growth of mold mycelium. After incubation at 25 to 30°C. for a few hours, during which the hyphae appear, the rice is again spread out in trays to a depth of about ½ in. and incubated until the period of maximum enzyme production

During incubation of the rice in the trays, it is necessary to regulate the humidity carefully, for an excess of moisture may lead to bacterial spoilage of the molded rice, while *insufficient moisture may not permit* proper development of the molds.

Some Mold Products.—Molds have been used for many centuries in such countries as Japan and China on account of the desirable changes which they produce in various substances. In the preparation of soybean sauces, sake, and various other products, molds, especially of the *A. flavus-oryzae* group, assume much importance

Soya sauce is a product manufactured from the soybean using *shoyu koji*. The strain of *A. flavus-oryzae* used should possess high proteolytic action, as well as amyloclastic, since there is very little starch in the beans used for the fermentations. The *koji* or starter is prepared by inoculating cooked soybeans, usually mixed with ground roasted wheat, with a selected strain of *A flavus-oryzae* and incubating until each bean is covered with sporulating mycelium. The *koji* is placed in a concentrated sodium chloride brine wherein an enzymic digestion takes place over a long period of time—from a few months to a few years. Daily agitation *and aeration are advantageous* Yeasts and bacteria aid in bringing about the changes in the bean The mash which results is dark brown in color and of thick consistency. The mash is finally pressed, and the juice or sauce is boiled, filtered, and processed. Soya sauce is used in the manufacture of table sauces or it may be employed directly.

Chinese Soya Sauce.—The production of Chinese soya sauce has been described by Lockwood,[2] of the Northern Regional Research Laboratory, U.S. Department of Agriculture. Dr. Lockwood was aided in his research by Mr. Pei Sung King, National Bureau of Industrial Research, Chunking, China, while the latter was working as a guest at the laboratory.

The raw materials used in the process consist principally of soya beans

[1] MORIKAWA, K., ". ⸺ ⸺ Alcohol Fermentation," doctor's dissertation in biology, ⸺ ⸺ Cambridge, 1926
[2] LOCKWOOD, L B , ⸺ ⸺

with smaller amounts of rice, coarsely ground parched wheat, and table salt.

There are three types of microorganisms used in the fermentation: two strains of *Aspergillus oryzae* (NRRL 1988 and 1989), a yeast (*Zygosaccharomyces soyae*, or *Hansenula* NRRL Y1096), and a bacterial culture (*Lactobacillus delbrueckii* NRRL B445).

In the production of Chinese soya sauce, there are two main steps. The first of these is concerned with the production of five cultures or *kojies* (two mold, one yeast, one bacterial, and one combined or soya koji); the second, with the brine fermentation of cooked soybeans.

PREPARATION OF THE KOJIES.—The medium used for the preparation of the mold *kojies* is rice that has been cooked until soft in boiling water, drained, divided into 2 portions and sterilized One portion of the sterilized rice is inoculated with *A. oryzae* NRRL 1988, the other with *A. oryzae* NRRL 1989. The inoculated rice is incubated until it is covered with green mold growth which requires 3 to 5 days. According to Lockwood, only tested strains of *A. oryzae* should be used in order to avoid the chance of producing sauces of poor quality

The medium for the yeast and bacterial *kojies* is prepared from soybeans [1] The soybeans are soaked overnight in water and drained Fresh water, 1 qt. for each 3 oz. of soybeans, is added, and the soybeans are cooked at a steam pressure of 15 lb for 1 hr. The broth thus obtained is dispensed into bottles and sterilized at a pressure of 15 lb for 15 min. The cooled broth is inoculated with a suitable culture of *Z. soyae* or of *Hansenula* NRRL Y1096 and incubated at 30 to 35°C (85 to 95°F) for 1 to 4 days, after which time it may be used. A 2-day-old culture may be stored in a refrigerator for several weeks before use, according to Lockwood [2] The bacterial *koji* is prepared by growing *L. delbrueckii* NRRL B445 in another portion of the soybean broth at a temperature of 35 to 40°C. This culture also may be used when 1 to 4 days old.

Soybeans are the main ingredient used in preparing the soya *koji* Five pounds of them are soaked in 1 gal. of water for 20 hr , after which the excess water is drained off The soybeans are cooked for 3 hr at a steam pressure of 15 lb Then 2 lb. 3 oz of coarsely ground parched wheat are added to the cooked soybeans and thoroughly mixed therewith. This mixture is spread in layers 2 in deep on suitable wooden or metal trays The four *kojies* (two mold, one yeast, and one bacterial) are mixed with the soybeans on the trays, after which the trays are stacked in such manner as to permit good circulation of air and incubated at 30°C. (or

[1] Lockwood, L B , *Soybean Digest,* **7** (No 12): 10–11 (1947)

[2] Ibid

85°F.) for 4 to 5 days. Under these conditions the soybeans become covered with a growth of *A. oryzae* and ready for the ensuing brine fermentation.

THE BRINE FERMENTATION.—The soya *koji*, prepared as outlined above, is placed in a suitable deep container and 1 gal. of brine, containing 2 lb. 1 oz. of table salt, is added for each 5 lb. (original weight) of soybeans used. The mash is incubated for 30 to 90 days at a temperature of 35 to 38°C. (or 95 to 100°F.) and then strained. The residue is pressed and the extract is combined with the strainings. The liquor thus obtained is heated to just below the boiling point for 20 min. Alum (1 oz. to 80 gal. or to 700 to 800 lb.) or kaolin (1 oz. to 1 gal.) is added to the heated liquor. The mixture is permitted to settle overnight after which it is filtered. The soya sauce obtained (12.5 lb. from 5 lb. of soybeans) is of the first or highest grade. A second-grade sauce is obtained by adding hot water to the press cake (1.25 gal. to that from 5 lb. of soybeans), mixing thoroughly, draining, and pressing the cake once more. Clarification of this liquor is achieved in the same manner as outlined for the first grade soya sauce. Then 0.75 lb. of table salt is added for each 5 lb. (original weight) of soybeans used. A yield of 10 lb. of second-grade soya sauce may be obtained from each 5 lb. of soybeans. The first and second grades of sauces may be mixed and caramel may be added, if desired, to produce a darker color and to increase the viscosity.

Tamari is a sauce prepared from soybeans, often with the addition of other materials such as rice. The flavor of *tamari* sauce differs from that of soya sauce. This difference in flavor is due to the use of *A. tamarii*, this mold being the dominant microorganism in this process. The fermentation period is shorter than that of the soya-sauce fermentation.

Miso is the name given to products prepared from cooked soybeans to which an *Aspergillus* starter and salt have been added and ripening permitted. There are several different types of *miso*. The concentration of the salt used, the flavoring in the ingredients employed, and the activity of the mold affect the type of final product. *Miso* is used especially as a breakfast food for children.[1]

The Uses of Mold Enzymes.—Four principal types of mold enzymes may be used industrially: amylases (diastases), invertase, proteases, and pectinase.[2] Amylases, of which there are at least two kinds, alpha-amylase (dextrinogenic) and beta-amylase (saccharogenic), and which may be produced from either molds or bacteria, are used in the preparation of sizes and adhesives, in the desizing of textiles, for the removal of

[1] RAMSBOTTOM, J., *Brit. Assoc. Advancement Sci.*, *Annual Rept*, 1936.
[2] WILLAMAN, J. J., Abstracts of Communications, Third International Congress of Microbiology, New York, p 335, Sept. 2–9, 1939.

starch from apple pomace in the manufacture of pectin, in the pharmaceutical trade, and for other purposes. In each of these cases the action of the amylases is initially upon starch, and, after the desired conversion of this substance has taken place, the enzymes may be destroyed by the application of heat.

Invertase, which may be produced from yeasts or molds, is used in the confectionery industry for the making of soft centers in chocolate-coated candies, for this enzyme converts sucrose to a mixture of glucose and fructose. This enzyme may also be used in the making of non-crystallizable sirups from sucrose, by a partial hydrolysis of this sugar.

Proteases, which may be obtained from *A. flavus*, or from bacteria, are used also for several purposes. They may be used in the degumming of silk goods, in the unhairing and bating of hides, in the manufacture of liquid glue, as a substitute for or combined with soap in the laundry business, and as an agent in the ripening of cheese. In the preparation of liquid glue, proteases partially hydrolyze the glue. Proteases are said to be the best agent used for this purpose in the preparation of liquid glue,[1] as controlled operations may thus be carried out. Proteases may also be used in making chillproof beer.

The term "proteases," as used, refers to a mixture of proteolytic enzymes, which may include true proteinases, and peptidases or creptases (polypeptidases, dipeptidases).[2]

Pectinase,[1] usually from penicillia,[2] may be used to aid in the clarification of fruit juices. Enzymes that hydrolyze pectin are also important in retting processes, as in the manufacture of linen from flax.

An enzyme "kinase" was found by Kunitz,[3] which had the ability to convert trypsinogen to trypsin in an acid medium. This enzyme was produced by a species of *Penicillium*.

References on Mold Enzyme Preparations, Mold Products, and Uses of Mold Enzymes

BOURGUIGNON, N. Enzymes Preparation and Application, *Tibia*, **15**: 13, 85 (1937).

CHURCH, M. B. Soy and Related Fermentations, *U-S Dept. Agr.*, Bull. 1152, 1923.

HARADA, T. Preparation of *Aspergillus oryzae* Enzymes, *Ind. Eng. Chem.*, **23**: 1424 (1931).

KELLNER, O., Y. MORI, and M. NAGAOKA: Researches on the Manufacture, Composition and Properties of "Koji," *Bull. Coll. Agr. Imp. Univ. Tokyo*, **5**: 9 (1888).

KITANO, T. Taka-amylase: Purification and Activity, *Jour. Soc. Chem. Ind. (Japan)*, **40**: 37–43B (1937).

KUNITZ, M., Formation of Trypsin from Trypsinogen by An Enzyme Produced by a Mold of the Genus *Penicillium, Jour. Gen. Physiol.*, **21**: 601 (1938).

---

[1] *Ibid*
[2] WALLERSTEIN, L., *Ind. Eng. Chem.*, **31**: 1218 (1939).
[3] KUNITZ, M., *Jour. Gen. Physiol.*, **21**: 601 (1939).

NISHIMURA, S : Zur Kenntnis der Takadiastase, *Chem. Zelle Gewebe*, 12: 202 (1925).

OPPENHEIMER, C.: "Die Technologie der Fermente," Georg Thieme Verlag, Leipzig, 1929

OSHIMA, K., and M. B. CHURCH: Industrial Mold Enzymes, *Jour. Ind Eng Chem*, 15: 67 (1923).

RAMSBOTTOM, J · The Uses of Fungi, *Brit Assoc. Advancement Sci.*, *Annual Rept*, 1936

SMITH, H. F., and W L OROLN: "Industrial Microbiology," The Williams & Wilkins Company, Baltimore, 1930.

TAKAMINE, J.: Enzymes of *Aspergillus oryzae* and the Application of Its Amyloclastic Enzyme to the Fermentation Industry, *Jour. Ind. Eng Chem*, 6: 824 (1914); *Chem News*, 109: 215 (1911).

TAKAMINE, J, JR., and K. OSHIMA: The Properties of a Specially Prepared Enzymic Extract, Polyzime, Comparing Its Starch Liquefying Power with Malt Diastase, *Jour. Am. Chem. Soc.*, 42: 1261 (1920).

THAYSEN, A. C , and L D. GALLOWAY: "Microbiology of Starch and Sugars," Oxford University Press, New York, 1930

THOM, C , and M B. CHURCH. "The Aspergilli," The Williams & Wilkins Company, Baltimore, 1926

UNDERKOFLER, L A , E. I. FULMER, and L. SCHOENE: Saccharification of Starchy Grain Mashes for the Alcoholic Fermentation Industry, *Ind Eng. Chem.*, 31: 734 (1939).

WAKSMAN, S. A , and W C DAVISOV: "Enzymes," The Williams & Wilkins Company, Baltimore, 1926

WALLERSTEIN, L . Enzyme Preparations from Microorganisms. Commercial Production and Industrial Application, *Ind Eng. Chem.*, 31: 1218 (1939).

WILLAMAN, J. J : Industrial Use of Microbial Enzymes, Abstracts of Communications, Third International Congress for Microbiology, New York, p 335, Sept 2–9, 1939.

Some Patents

BLOEDE, V. G · Process of Manufacturing Vegetable Glue, U S. Patents 1,257,307, Feb. 26, 1918, & 1,273,571, July 23, 1918

BOIDIN, A , and J EFFRONT. Process for Treating Amylaceous Substances, U S Patent 1,227,374, May 22, 1917

—— and ——: Process of Manufacturing Diastase and Toxins by Oxidizing Ferments, U S Patent 1,227,525, May 22, 1917.

—— and ——: Method of Producing Bacterial Enzyme Preparations, U S. Patent 1,744,742, Jan 28, 1930

DOUGLAS, R Process of Preparing Pectin, U S Patent 1,858,820, May 17, 1932

FRÄNKEL, S : Manufacture of Diastase, U S. Patent 1,129,387, Feb. 23, 1915

PAINE, H S., and J. HAMILTON: Process for Preparing Fondant or Chocolate Soft Cream Centers, U S Patent 1,437,816, Dec. 5, 1922

TAKAMINE, J : Preparing and Making Fermented Alcoholic Liquors, U S Patent 525,819, Sept 11, 1894

——: Preparing and Making Taka-Koji, U S Patent 525,820, Sept 11, 1894

——: Preparing and Making Moto, U S. Patent 525,821, Sept. 11, 1894

——: Preparing and Making Taka-Moyashi, U S. Patent 525,822, Sept. 11, 1894

——: Process of Making Diastatic Enzyme, U S Patent 525,823, Sept. 11, 1894

———. Taka-Koji Ferment and Process of Making the Same, U S Patent 525,824, Sept. 11, 1894.

———: Means for and Method of Converting Starch Material into Sugar, U S Patent 525,971, Sept. 11, 1894.

———: Process of Converting Starchy Material into Sugar, U.S. Patent 562,103, June 16, 1896.

———: Enzym, U.S. Patent 991,560, May 9, 1911

———: Amylolytic Enzym, U S Patent 991,561, May 9, 1911

———: Process for Producing Diastatic Product, U S Patent 1,054,324, Feb. 25, 1913

———: Diastatic Product, U S Patent, 1,054,626, Feb 25, 1913

———: Diastatic Product and the Process for Producing the Same, U S Patent 1,148,938, Aug 3, 1915

———: Process for Producing Diastatic Product, U S Patent 1,263,817, Apr. 23, 1018

——— and J Takamine, Jr : Enzymic Substance and Process of Making the Same, U.S Patents 1,391,219, Sept 20, 1921, and 1,460,736, July 3, 1023

Wallerstein, L : Beer and Method of Preparing Same, U.S Patent 995,820, June 20, 1911

———: Preparation for Use in Brewing, U.S Patent 995,823, June 20, 1911

———: Method of Treating Beer or Ale, U.S. Patents 995,824, 995,820, June 20, 1911.

———: Method of Treating Beer or Ale, U S Patent 997,873, July 11, 1911

———: Invertase Preparation and Method of Making the Same, U S Patents 1,019,075, 1,010,076, July 25, 1933

——— Process of Maturing and Ripening Beers and Ales, U S Patent 2,077,446, Apr 20, 1037

——— Process of Chillproofing and Stabilizing Beers and Ales, U S Patents 2,077,-447,2,077,448, Apr 20, 1937

——— Process of Chillproofing and Stabilizing Malt Beverages, U S Patent 2,077,-449, Apr 20, 1937

———. Rubber, U S Patent 2,097,481, Nov 2, 1937

———. Deproteinization of Rubber Latex, U S Patent 2,116,089, May 3, 1938

Wallerstein, M Process of Making Chocolate Sirup, U.S Patents 1,854,353, 1,854,355, Apr 19, 1032.

CHAPTER XXXIV

THE PRODUCTION OF FAT BY MOLDS

Historical.—In 1906, Browne[1] reported the results of some analyses made on the dried mycelium of a species of *Citromyces* (*Penicillium*) that had grown as a scum on the surface of tanks of leftover molasses in a hot room of a sugar factory. The mycelium contained 27.50 per cent of fat, which in several physical and chemical ways resembled butterfat, for example, in respect to the saponification number, the Reichert-Meissl number, the melting point, and the iodine number. The fat differed, however, from butterfat in other respects, for example, in the acid number, and the mean molecular weight of the soluble and the mean molecular weight of the insoluble acids. The fat contained a preponderance of caproic (CH_3 (CH_2)$_4$·COOH) and caprylic ($CH_3(CH_2)_6$ COOH) acids.

Oleic, palmitic, and two unidentified acids were detected by Sullivan[2] in the alcoholic soda extract of the dried mycelium of *Penicillium glaucum*, that had been grown on Raulin's medium.

Belin[3] (1926) mentioned fat production by the genus *Aspergillus*.

Rockwell and O'Flaherty[4] (1931), during a study of the physiology of some of the common molds, analyzed the mycelia of some of them. They discovered 0.58 per cent of fat (by ether extraction) in the moist mycelium of *Aspergillus niger*.

Barber[5] investigated fat production by a green species of *Penicillium*. The species appeared to grow equally well on nutrient solutions of sucrose, glucose, or xylose, but not as well on glycerol. In each case, the same fat or mixture of fats was apparently produced. The ether extract of the mycelia contained palmitic, stearic, oleic, alpha-linolic, and beta-linoleic

[1] BROWNE, C A., JR., *Jour. Am. Chem. Soc.*, 28: 465 (1906).

[2] SULLIVAN, M X , *Science*, 38: 678 (1913).

[3] BELIN, P., *Bull. soc. chim biol* , 8: 1031 (1926).

[4] ROCKWELL, G. E., and F. O'FLAHERTY, *Jour. Am Leather Chem Assoc* , 26: 216 (1931).

[5] BARBER, H., *Jour Soc. Chem. Ind. (Trans)*, 46: 200T (1927); *Biochem. Jour* , 33: 1158 (1929).

acids, free and as glycerides. Sterols were also found. A yield of 14 per cent fat was obtained from the dried mycelium of the mold when grown on a 5 per cent sucrose solution.

The energy relations involved during the production of fat from sugar by *A. niger* were studied by Terroine and Bonnet[1] (1927).

The relation of the temperature at which the molds were grown to the degree of unsaturation of the fatty acids produced was studied by Pearson and Raper.[2] Using the iodine number as a measure of the degree of unsaturation, they showed that both *A. niger* and *Rhizopus nigricans* produced more unsaturated acids at low temperatures than at higher temperatures

Pontillon[3] studied the effect of the constituents of the medium, inorganic and organic, on the quality and quantity of fat produced by *A. niger*

THE NATURE OF THE FATS PRODUCED BY MOLDS

Lipids of Penicillium javanicum van Beijma.—The mold was cultivated on a 20 per cent nutrient glucose solution at 30°C, and the dried mycelium was extracted with a redistilled petroleum ether, yielding 11 per cent of oil[4] Physical and chemical characteristics of the oil are shown in the accompanying table.

TABLE 137.—PHYSICAL AND CHEMICAL CHARACTERISTICS OF OIL FROM *P. javanicum*[1]

Solidification point, °C.	6–7
Melting point, °C	about 15
Specific gravity (25°/25°)	0 9145
Refractive index (25°)	1 4680
Acid value	10 6
Saponification value	191
Iodine value (Hanus)	84 0
Reichert-Meissl value	0 3
Acetyl value	10 7
Unsaponifiable matter, per cent	2 00
Saturated acids (corrected), per cent	30 8
Unsaturated acids (corrected), per cent	60 8
Melting points of mixed saturated acids, °C	52 5
Mean molecular weight of saturated acids	272

[1] WARD, G E., and G S JAMIESON, *Jour Am Chem Soc*, **56**: 973 (1934).

[1] TERROINE, E. F., and R BONNET, *Bull soc chim biol*, **9**: 588 (1927).

[2] PEARSON, L. K., and H S RAPER, *Biochem Jour.*, **21**: 875 (1927)

[3] PONTILLON, C, *Rev gén. botan.*, **44**: 465, 526 (1932), **45**: 20 (1933).

[4] WARD, G. E., and G S JAMIESON, *Jour. Am Chem. Soc.*, **56**: 973 (1934).

Analyses of the unsaturated and saturated fractions follow:

TABLE 138.—ANALYSIS OF UNSATURATED FRACTION[1]

	In unsaturated fraction, per cent	In oil	
		Acid, per cent	Glyceride, per cent
Oleic acid	52.1	31.7	33 2
Linoleic acid..	47.9	29.1	30.5
Total	100.0	60 8	63.7

[1] WARD, G. E., and G. S. JAMIESON, *Jour. Am. Chem. Soc.*, **56**: 973 (1934).

TABLE 139.—ANALYSIS OF THE SATURATED FRACTION

	In saturated fraction, per cent	In oil	
		Acid, per cent	Glyceride, per cent
Palmitic acid .	69.5	21.4	22 4
Stearic acid . .	28.0	8.6	9.0
Tetracosanic acid	2 5	0.8	0 8
Total ..	100 0	30.8	32.2

Lipids of Aspergillus sydowi.—The alcohol-ether extract of the dried mycelium of *A. sydowi* contained oleic, linoleic, palmitic, stearic, and *n*-tetracosanic acids; glycerol; and sterols as shown in the following table:

TABLE 140.—COMPOSITION OF THE SIMPLE LIPIDS OF *A. sydowi*[1,2]

Fatty acids	80 8
Volatile acids (calculated as butyric).	0 46
Saturated acids. .	22 6
Palmitic	8 8
Stearic	11.0
N-Tetracosanic	0 9
Unsaturated acids	52 9
Oleic.. .	29 6
Linoleic	16 3
Higher acids	1 7
Unsaponifiable . .	8 18
Total sterols[3]	5 36
Glycerol . .	4 2

[1] STRONG, F. M., and W. H. PETERSON, *Jour. Am. Chem. Soc.*, **56**: 952 (1934).
[2] Figures indicate percentage of the original lipids
[3] Based on the colorimetric sterol determination.

Lipids of Penicillium aurantio-brunneum.[1]—The mold was grown on a glucose-inorganic salt solution in the manner described by Peterson

TABLE 141.—APPROXIMATE COMPOSITION OF THE SIMPLE LIPIDS

	Per Cent
Total fatty acids	85.4
Oleic[1]	40 2
Linoleic[1]	31 2
Palmitic[2]	8 6
Stearic[2]	5 3
Unsaponifiable	4 5
Ergosterol	1 9
Glycerol	3 1

[1] Calculated from the weight and iodine number of the unsaturated acids
[2] Calculated from the weight and neutral equivalent of the crude saturated acids.

and his associates. The mycelial mats[2] were steamed to destroy the mold, dried at 65°C., ground finely, and extracted with a 1:1 mixture of alcohol and ether. The dried mycelium yielded 11.6 per cent crude lipids.

Table 141 shows the approximate composition of the simple lipids, which were mainly the glycerides of palmitic, stearic, oleic, and linoleic acids.

The Lipid Content of Molds.—The lipid content of different molds varies considerably. On the basis of the dry weight, the mycelium may contain as much as 41.5[3] or as little as 1 per cent of lipid Mold spores have been reported to contain from 1 to 14 per cent of lipid.[4]

Pruess, Eichinger, and Peterson cultivated 24 molds on 2 different types of media: (1) a glucose-inorganic salts medium containing calcium carbonate in excess and (2) a glucose-malt-sprouts medium Results of the analyses of the dried mycelia of the molds are shown in Table 142. The lipid content of the mycelia varied from 1 1 to 19 9 per cent, with an average of 6.0 per cent, when the molds were grown on the glucose-inorganic salts (synthetic) medium; and from 1 5 to 21 4 per cent, with an average of 8 8 per cent, when the molds were grown on the glucose-malt-sprouts (organic) medium The average lipid content of all the molds was over 46 per cent greater when grown on the organic medium than when grown on the synthetic medium. However, some molds, for

[1] KROEKER, E H, F M STRONG, and W H PETERSON, *Jour. Am. Chem. Soc.*, 57 : 354 (1935)

[2] PETERSON, W H, L M PRUESS, H J GORCIA, and H C GREENE, *Ind. Eng Chem*, 25 · 213 (1933)

[3] WARD, G E, L B LOCKWOOD, O E MAY, and H T HERRICK, *Ind Eng Chem*, 27 : 318 (1935).

[4] PRUESS, L M, E C EICHINGER, and W H PETERSON, *Centr Bakt Parasitenk*, Abt II, 89 : 370 (1934)

TABLE 142—COMPOSITION OF CERTAIN MOLDS[1]

Organism	Glucose-inorganic-salts medium							Glucose-malt-sprouts medium				
	Weight of dry pad grams per 100 cc	Sterol, per cent	Lipid, per cent	Crude protein (N × 6.25), per cent	Carbohydrate (by difference), per cent	Free fatty acid of lipid (as oleic), per cent	Weight of dry pad, mg. per 100 cc	Sterol, per cent	Lipid, per cent	Crude protein (N × 6.25), per cent	Carbohydrate (by difference), per cent	Free fatty acid (as oleic), per cent
Aspergillus clavatus 4700A	2.67	0.65	4.7	34.4	60.2	50.3	55	0.58	6.1	25.5	68.2	8.3
Aspergillus carbonarius 4030 B	4.04	0.29	4.1	13.7	87.6		25	0.30	4.1	15.5	80.2	73.1
3694 B	3.63	0.42	3.9	25.0	81.0		16	0.34	1.5	12.4	86.0	16.0
3510	3.63	0.30	3.6	17.6	75.7	20.0	37	0.34	0.7	14.3	72.9	15.0
	3.04	0.50	4.0	28.6	64.7	15.2	40	0.77	1.0	13.4	80.2	
Aspergillus nidulans 245	2.89	0.82	3.9	36.3	57.6	53.0	84	0.48	3.0	28.1	60.5	
	2.83	0.10	3.8	31.2	65.0	13.4	63	0.46	2.7	24.1	70.7	36.2
Aspergillus niger 910	2.83	0.18	2.9	28.7	68.6		94	0.32	5.7	27.0	61.3	10.9
8942	3.10	0.32	3.5	40.5	55.0	44.9	31	1.05	4.8	20.7	73.4	
	3.28	0.38	4.1	32.0	63.5	29.7	53	0.54	3.8	22.7	72.9	24.3
Aspergillus ochraceus 4700A	3.56	0.55	2.5	25.0	69.3		16	0.31	3.5	13.1	79.1	18.0
Aspergillus oryzae 905	2.17	0.84	5.1	43.7	49.4	19.7	98	0.09	5.2	11.3	79.1	38.5
Aspergillus achraceus no 3536a	1.77	0.78	5.8	32.9	61.3	34.2	46	0.07	5.2	38.1	56.7	20.7
	2.02	0.87	4.5	27.5	64.5	44.9	56	0.55	3.2	18.3	77.8	30.8
	1.66	0.81	5.5	23.7	54.0	67.8	21	0.74	1.4	20.6	74.2	30.4
	1.75	0.40	4.8	36.3	55.5		31	0.33	1.4	15.9	82.1	
	1.64	0.25	4.9	30.0	65.1	39.4	48	0.00	10.1	26.9	63.0	41.2
												27.4
	2.41	0.75	14.0	31.6	62.5	27.6	2.92	0.78	8.8	22.5	69.6	26.3

[1] C. Eichinger and W. H. Peterson, Centr. Bakt. Parasitenk., Abt. II, 89: 370 (1934).

at: it s varies from 2.5 to 8.5 per cent.

whit: it s varies from 10 to 4.5 per cent.

example, *Aspergillus nidulans* 1 and *Paecilomyces rariota* 1, yielded more lipid on the latter medium than on the former medium.

Ward and his associates extracted the crude fat from 61 different molds, 39 penicillia and 22 aspergilli, using ethyl ether. Of these molds, 10 contained more than 15 per cent, but only 6 more than 20 per cent of crude fat, as indicated in Table 143.

TABLE 143.—SOME MOLDS CONTAINING MORE THAN 15 PER CENT OF CRUDE FAT[1]

Mold	Crude Fat in Dried Mycelium. Per Cent
Penicillium bialouiezense	17 0
P. citrinum Thom	18 1
P. hirsutum Dierckx	18 4
P. soppi Zal	20 2
P. javanicum van Beijma	22 2
P. roqueforti Thom	22 9
P. oxalicum Currie and Thom	24 4
P. piscarum Westling	26–28
P. flavocinerum Bourge	28 5
Aspergillus flavus Thom and Church	16 0

[1] WARD, LOCKWOOD, MAY, and HERRICK, *loc. cit.*

FACTORS AFFECTING THE LIPID CONTENT OF MOLDS

Sufficient data have already been presented to indicate the importance of the species of mold in respect to the lipid content. Cultural conditions are likewise important: the concentration of the sugar, the kind and quantity of nitrogen-containing substance, the presence of small quantities of materials that stimulate fat production, the pH, the temperature, and the incubation period. The effect of cultural conditions on two molds *Penicillium javanicum* van Beijma and *Aspergillus fischeri* are discussed in the succeeding paragraphs.

Fat Production by Penicillium javanicum van Beijma.[1]—This mold was selected for study since it produced the heaviest mats, yielding a considerable amount of lipid. The mold was described by Beijma.[2]

The mold was cultivated in 200-cc. Pyrex Erlenmeyer flasks, into each of which were placed 75 cc. of the following medium.

	Grams per Liter
Glucose	200
NH_4NO_3	2 25
KH_2PO_4	0 3
$MgSO_4 \cdot 7H_2O$	0 25

[1] LOCKWOOD, L. B., G. E. WARD, O. E. MAY, H. T. HERRICK, and H. T. O'NEILL, *Centr. Bakt. Parasitenl.*, Abt. II, 90: 411 (1934)

[2] BEIJMA THOE KINGMA, F. H. VAN, *Verhandel. Akad. Wetensch. Amsterdam, Afdeel. Natuurkunde*, 26: (4) 16 (1929)

A chemically pure glucose (anhydrous) was used when the effect of the ions of various salts was ascertained, otherwise a commercial grade of glucose containing 91.5 per cent of pure glucose, 8 per cent water, and 0.4 per cent dextrin was used.

The pH of the medium was 4 to 5; the temperature of incubation, 30°C.; and the period of incubation 12 days.

In the following table is shown the effect of varying the glucose concentration:

TABLE 144.—THE EFFECT, ON FAT PRODUCTION, OF VARYING THE GLUCOSE
CONCENTRATION[1]

Glucose, per cent	Mat weight, per cent	Fat, per cent	Culture acid, equivalent cc. N/10 acid	Glucose consumed, grams
20	2 522	29 0	149	10 3
30	2 400	34 6	209	11.3
40	1 964	41 5	148	9.9
50	1 021	35 2	20	5 2

[1] LOCKWOOD, L. B., G. E. WARD, O. E. MAY, H. T. HERRICK, and H. T. O'NEILL, *Centr. Bakt. Parasitenk.*, Abt. II, 90: 411 (1934)

The greatest mat weight occurred when the glucose concentration was 20 per cent; the greatest titrable acidity at 30 per cent; and the greatest percentage of fat at 40 per cent.

Xylose, galactose, maltose, sucrose, glycerol, starch, and dextrin yield lipids, xylose being a particularly good source of carbon.

P. javanicum utilized sodium, potassium, magnesium, or calcium nitrates; sodium nitrite; ammonium chloride or ammonium sulphate as sources of nitrogen, but ammonium nitrate was the best source. Ammonium chloride and ammonium sulphate caused the pH to drop too low.

The growth and metabolism of *P. javanicum* were favored by the presence of 2.25 to 3.375 g of NH_4NO_3, 0.3 to 1.2 g. of KH_2PO_4, and about 0.25 g. of $MgSO_4 \cdot 7H_2O$ per liter of medium.

Chromic, columbic, ferric, molybdic, and tungstic ions stimulated mycelial growth, fat and citric acid formation, and glucose consumption.

The pH range of 3 1 to 6.8 was favorable, the final pH of the medium being about 2.5 in each case.

Increasing the air pressure within the culture flask tended to inhibit the metabolism of *P. javanicum*.

Decreasing the ratio of surface area to volume decreased the efficiency of the conversion of glucose to lipids

The production of fat from glucose by *P. javanicum* in a large-scale

laboratory apparatus is described by Ward, Lockwood, May, and Herrick in a later report.[1]

The composition of two media used for growing molds for lipid production, known as "*G solution*" and "*M solution*," of which *M* solution gave the more consistent results, follows.[1]

TABLE 145.—SOME MEDIA USED FOR GROWING MOLDS FOR LIPID PRODUCTION

G solution		M solution	
Substance	Grams per liter	Substance	Grams per liter
Commercial glucose	220	Commercial glucose	220
NH_4NO_3	4 50	NH_4NO_3	2 25
KCl	0 40	KH_2PO_4	0 3
H_3PO_4	0 216	$MgSO_4 \cdot 7H_2O$	0.25
$MgSO_4 \cdot 7H_2O$	2 00		

Fat Production by Aspergillus fischeri.—Prill, Wenck, and Peterson have studied the effect of various factors, such as glucose concentration, ammonium nitrate concentration, acidity, alkalinity, temperature, neration, and incubation period, on fat production by *A. fischeri*.[2]

The mold was cultured at 30°C in 500-cc. Pyrex Erlenmeyer flasks, each of which contained 100 cc of the following solution, except where the nature of the experiment required otherwise

	Grams
Commercial glucose (cerelose)	20
NH_4NO_3	1 00
KH_2PO_4	0 68
$MgSO_4 \cdot 7H_2O$	0 50
$FeCl_3 \cdot 6H_2O$	0 016
$ZnSO_4 \cdot 7H_2O$	0 005
Distilled water, to make 100 0 cc	
$CaCO_3$	

The mold growth was destroyed at the end of the incubation period by autoclaving it at 120°C for 10 min. Mycelial mats were then washed with water, dried for 2 days at 37°C., ground, and extracted with hot absolute alcohol for 12 hr. or more.

A high fat content[2] in the mycelium was favored by a high concentration of glucose, a low concentration of ammonium nitrate (NH_4NO_3),

[1] WARD, LOCKWOOD, MAY, and HERRICK, *loc. cit*

[2] PRILL, E A, P R. WENCK, and W H PETERSON, *Biochem Jour*, 29: 21 (1935).

[3] *Ibid*

and a neutral or slightly alkaline solution. (Compare with fat production by *Endomyces vernalis*.) For further details consult the paper by Prill, Wenck, and Peterson.[1]

References on Production of Fat by Molds

BARBER, H. H.: The Production of Fat by a Species of Penicillium Grown in Sucrose Solution, *Jour. Soc. Chem. Ind.* (*Trans.*) 46: 200T (1927).

————: Production of Fat from Carbohydrates and Similar Media by a Species of Penicillium, *Biochem. Jour.*, 33: 1153 (1929).

BEIJMA THOE KINGMA, F. H. VAN: Mykologische Untersuchungen-*Penicillium javanicum* nov. spec., *Verhandel. Akad. Wetenschappen Amsterdam, Afdeel. Naturkunde*, 26 (4), 16 (1929).

BELIN, P.: *Bull. soc. chim. biol.*, 8: 1081 (1926).

BROWNE, C. A., JR.: The Fermentation of Sugar-cane Products, *Jour. Amer. Chem. Soc.*, 28: 465 (1906).

FINK, H. H., H. HAEHN, und W. HOERBURGER: Über die Versuche zur Fettgewinnung mittels Mikroorganismen mit besonderer Berücksichtigung der Arbeiten des Instituts für Gärungsgewerbe, *Chem. Ztg.*, 61: 689-693; 723-726, 744-747 (1937). (A very comprehensive article with 52 references on fat production by microorganisms. See also Chap. IX.)

KNOCKER, C. H., F. M. STRONG, and W. H. PETERSON: The Chemistry of Mold Tissue. VII. The Lipids of *Penicillium Aurantio-Brunneum*, *Jour. Am. Chem. Soc.*, 57: 354 (1935).

LOCKWOOD, L. B., G. E. WARD, O. E. MAY, H. T. HERRICK, and H. T. O'NEILL: The Production of Fat by *Penicillium javanicum* van Beijma, *Centr. Bakt. Parasitenk.*, Abt. II, 90: 411 (1934).

PEARSON, L. K., and H. S. RAPER: The Influence of Temperature on the Nature of Fat Formed by Living Organisms, *Biochem. Jour.*, 21: 875 (1927).

PETERSON, W. H., L. M. PRUESS, H. J. GORCIA, and H. C. GREENE: Large-scale Laboratory Cultivation of Molds, *Ind. Eng. Chem.*, 25: 213 (1933).

PONTILLON, C.: Contribution à l'étude physiologique des lipides du *Sterigmatocystis nigra*, *Rev. gén. botan.*, 44: 465, 526 (1932), 45: 20 (1933).

PRILL, E. A., P. R. WENCK, and W. H. PETERSON: The Chemistry of Mould Tissue. VI. Factors Influencing the Amount and Nature of Fat Produced by *Aspergillus fischeri*, *Biochem. Jour.*, 29: 21 (1935).

PRUESS, L. M., E. C. EICHINGER, and W. H. PETERSON: The Chemistry of Mold Tissue. III. Composition of Certain Molds with Special Reference to the Lipid Content, *Centr. Bakt. Parasitenk.*, Abt. II, 69: 370 (1934).

ROCKWELL, G. E., and F. O'FLAHERTY: Studies in the Physiology of Moulds. II. Composition and Culture of Moulds. *Jour. Am. Leather Chem. Assoc.*, 26: 216 (1931).

STRONG, F. M., and W. H. PETERSON: Chemistry of Mold Tissue. IV. The Lipids of *Aspergillus Sydowi*, *Jour. Am. Chem. Soc.*, 56: 952: 1934.

SULLIVAN, M. X.: Some Organic Constituents of the Culture Solution and the Mycelium of Molds from Soil, *Science*, 38: 678 (1913).

TERROINE, E. F., and R. BONNET: L'energie de croissance X.—Formation des matières grasses au dépens de glucides chez les microorganisms, *Bull. soc. chim. biol.*, 9: 588 (1927).

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[1] *Ibid.*

WARD, G. E , and G. S. JAMIESON: The Chemical Composition of the Fat Produced by *Penicillium javanicum* van Beijma, *Jour. Am. Chem. Soc*, 56: 973 (1934).

——, L. B. LOCKWOOD, O. E. MAY, and H. T. HERRICK: Production of Fat from Glucose by Molds.   Cultivation of *Penicillium javanicum* van Beijma in Large-scale Laboratory Apparatus, *Ind. Eng Chem.*, 27: 318 (1935).

WOOLLEY, D. W., F. M. STRONG, W. H PETERSON, and E. A PRILL: The Chemistry of Mold Tissue   X.   The Phospholipides of *Aspergillus Sydowi*, *Jour. Am. Chem. Soc.*, 57: 2589 (1935).

SOME MINOR CHEMICAL ACTIVITIES OF THE LOWER FUNGI

One of the most interesting aspects of microbiology is the biochemistry of molds. Because of their ubiquity and the mischief these organisms, once regarded as biological outcasts, can do in many branches of industry, their study has distinct economic importance. This study of the lower fungi has advanced rapidly since about 1922. The outstanding work of Prof. Raistrick and his associates is mentioned in particular, because of the new and careful methods of investigation they have devised. A large number of papers, many of which are cited at the end of this chapter, have been published on various aspects of this organized research.

A study of Table 120 (page 563) will give the reader some idea as to the diversified metabolic products formed by the lower fungi. Methods for production of some of the most important substances mentioned in this table have been discussed in some detail in the preceding chapters. The scope of this text does not permit extended consideration of others, but the interested reader will find excellent reviews of papers by Birkinshaw,[1] Clutterbuck,[2] Iwanoff and Zwetkoff,[3] Lockwood and Moyer,[4] Raistrick,[5] Tatum,[6] Porter,[7] Gould,[8] and others. Likewise at the end of this chapter there will be found a large number of references to the literature.

ACIDS

A large number of organic acids are produced by molds although at the present time only a few of them are of commercial importance. These have already been discussed. Brief mention will now be made of some other organic acids that are metabolic products of molds.

[1] BIRKINSHAW, J. H., *Biol. Rev.*, **12**: 357–391 (1937).

[2] CLUTTERBUCK, P. W., *Jour Soc. Chem. Ind*, **55**: 55T–61T (1936).

[3] _____ *Ann Rev Biochem*, **5**: 585–612 (1936).

[4] _____ *Rev.*, **4**: 140–164 (1935).

[5] _____ (1932), **7**: 316–349 (1938).

[6] TATUM, E. L., *Ann Rev. Biochem.*, **13**: 667 (1944).

[7] PORTER, J. R., "Bacterial Chemistry and Physiology," John Wiley & Sons, Inc., New York, 1946.

[8] GOULD, B. S., *Scientific Report, Series No. 7*, Sugar Research Foundation, Inc., New York, June, 1947.

TABLE 146—SOME ACIDS FORMED BY MOLDS

Acid	Formula	Produced by
Aconitic[69]* $(C_6H_6O_6)$	CH_2 $COOH$ C $COOH$ CH $COOH$	*Aspergillus itaconicus*
Byssochlamic[45] $(C_{18}H_{20}O_6)$		*Byssochlamys fulva*
Carlic (anhydrous)[46,43] $(C_{10}H_{10}O_6)$	O O—C—CH CO CH_2 CH_2 CH_3 OC CH CO CH_2	*Penicillium charlesii* G Smith
Carlosic[44,43] $(C_{10}H_{14}O_6)$	HOC==$C \cdot CO$ CH_2 CH_2 CH_3 $HOOC$ H_2C HC CO O	*P. charlesii* G Smith
Carolic (anhydrous)[44,43] $(C_{11}H_{16}O_4)$	O H_2C CH C==$C \cdot CO$ CH_2 CH_2 CH_3 O CO O	*P. charlesii* G. Smith
Carolinic[44,43] $(C_{11}H_{16}O_5 \cdot H_2O)$	HOC==C CO CH_2 CH_2 $COOH$ H_2C CH CO O	*P. charlesii* G Smith
Citric $(C_6H_8O_7)$	CH_2 $COOH$ $C(OH)$ $COOH$ CH_2 $COOH$	*A niger*, etc See Chap XXV
3 5-Dihydroxy-phthalic acid[53] $(C_8H_6O_6)$	$COOH$ $COOH$ HO OH	Some species and strains of the *P brevi-compactum* series
Dimethyl pyruvic[69] $(C_5H_8O_3)$	CH_3 CH_3—$C \cdot CO$ $COOH$ H	*A niger*

* The numbers in parentheses refer to the citations at the end of the chapter

TABLE 146.—SOME ACIDS FORMED BY MOLDS.—(Continued)

Acid	Formula	Produced by
l-Ethylene oxide α, β dicarboxylic ($C_4H_4O_5$)	HOOC·CH ＼ O ／ HC·COOH	Monilia formosa P. vinferum
Formic (CH_2O_2)	H·COOH	A. oryzae
Fulvic[44] ($C_{14}H_{12}O_6$)		P. griseo-fulvum Dierckx P. flexuosum Dale P. brefeldianum Dodge
Fumaric ($C_4H_4O_4$)	CH·COOH ‖ CH·COOH	Species of the genera Rhizopus, Mucor, etc.
Gallic (3·4·5-trihydroxybenzoic) $C_7H_6O_5$	COOH [benzene ring] HO OH OH	A. niger See Chap XXVIII
Gentisic[23] (2·5-dihydroxybenzoic) ($C_7H_6O_4$)	COOH [benzene ring] OH HO	P. griseo-fulvum Dierckx
Glaucic[23] ($C_{17}H_{22}O_5$)		A glaucus
Glauconic[23] I ($C_{18}H_{26}O_7$) II ($C_{18}H_{26}O_8$)		Some green Penicillium species
d-Gluconic ($C_6H_{12}O_7$)	COOH | HCOH | HOCH | HCOH | HCOH | CH₂OH	A. niger P chrysogenum, etc. See Chapter XXVI
Glucuronic[23] ($C_6H_{10}O_7$)	CHO | (CHOH)₄ | COOH	Ustulina vulgaris

TABLE 146.—SOME ACIDS FORMED BY MOLDS —*(Continued)*

Acid	Formula	Produced by
d-Mannonic ($C_6H_{12}O_7$)	COOH HOCH HOCH HCOH HCOH CH_2OH	P. purpurogenum var. rubrisclerotium

l-γ-Methyl-tetronic[45] ($C_5H_6O_3$)	HOC====CH
	H_2C·HC CO
	    \\ /
	    O
		P. charlesii G. Smith

6-Methylsalicylic[18,91] (2-Hydroxy-6-methylbenzoic) ($C_8H_8O_3$)	COOH H_3C OH	P. griseo-fulvum Dierckx P. flexuosum Dale
Miniolutein[34] ($C_{16}H_{22}O_7$)	CH_3 $(CH_2)_6$ CH————CO HO·C COOH CH————O COOH	P. minio-luteum Dierckx
Mycophenolic[21] ($C_{17}H_{20}O_6$)	HOC CO HOOC·C C C O H_3C·C H_3C·C C ·C_5H_{12}	Some species and strains of the P. brevi-compactum series
Oxalic ($C_2H_2O_4$)	COOH COOH	A. niger etc

Penicillic[48] ($C_8H_{10}O_4$)	CH_2 O·CH_3
	    C CO C=CH-COOH
	CH_2
	         ↥
	CH_2 OH OCH_3
	or
	    C·C C==CH CO
	CH_2
	      O
		P. puberulum Baurier P. cyclopium Westling

Succinic acid is produced by several species of *Mucor*, by a white species of *Aspergillus*, by *A. terreus*, *Penicillium aurantio-virens*, and *Fumago vagans*, and by other molds. Succinic acid may originate from cell proteins of from the fermentation of carbohydrates. Its exact origin is not entirely clear.

TABLE 147.—SOME MOLD PIGMENTS

		Vogu.[.,.]*
Aurofusarin (orange-yellow) ($C_{36}H_{22}O_{12}$)		*Fusarium culmorum*[11] (W. G. Smith) Sacc.
Auroglaucin (orange) ($C_{17}H_{22}O_3$)		Species of *Aspergillus glaucus* series[40]
Beta-carotene (yellow)		*Mucor hiemalis*[69] *Phycomyces blakesleanus*
Boletol (blue) ($C_{15}H_8O_7$)		*Boletus luridus*[87] *B. satanas* *B. strobilaceus*
Carviolacin ($C_{30}H_{21}O_6$)		*P. carminoviolaceum*[194]
Catenarin [β-(hydroxy-methyl)-1:5:8-trihydroxyanthraquinone] (red) ($C_{15}H_{10}O_6$)		*Helminthosporium catenarium*[17] Drechsler *H. gramineum* Rabenhorst *H. tritici-vulgaris* Nisikado *H. velutinum*
Chrysogenin (yellow) ($C_{15}H_{22}O_6$)		*Penicillium chrysogenum*[21]
Citrinin (yellow) ($C_{13}H_{14}O_5$)		*P. citrinum* Thom[16] *A. terreus*
Citromycetin (yellow) ($C_{14}H_{10}O_7 \cdot 2H_2O$)		Species of *Citromyces*[11] *C. glaber* *C. pfefferianus*

* Numbers in parentheses refer to bibliography at the end of the chapter

TABLE 147.—SOME MOLD PIGMENTS.—(*Continued*)

Pigment	Structural formula	Produced by
Cynodontin (probably 1:4:5:8-tetrahydroxy-2-methyl-anthraquinone) (bronze) ($C_{15}H_{10}O_6$)		*H avenae* Eidam[12] *H cynodontis* Marignoni *H euchlaenae* Zimmermann
4,5-Dihydroxy-7-methoxy-2-methyl-9-anthranol		*A glaucus*[42]
4,5-Dihydroxy-7-methoxy-2-methyl-10-anthranol		*A glaucus*[42]
Erythroglaucin (dark red) ($C_{16}H_{12}O_6$)		*A ruber*[40, 42] Species of *A glaucus* series
Flavoglaucin (lemon-yellow) ($C_{15}H_{22}O_2$)		Species of *A glaucus* series[40]
Fulvic acid (yellow) ($C_{14}H_{10}O_6$)		*P brefeldianum*[44] Dodge *P flexuosum* Dale *P griseo-fulvum* Dierckx
Funiculosin ($C_{15}H_{10}O_5$)		*P funiculosum*[50]
Helminthosporin (2-methyl-4:5:8-trihydroxyanthraquinone) (dark-maroon crystals) ($C_{15}H_{10}O_5$)		*H gramincum* Rabenhorst[35] *H catenarium* Drechsler *H cynodontis* Marignoni *H tritici-vulgaris* Nisikado

TABLE 147.—SOME MOLD PIGMENTS.—(Continued)

Pigment	Structural formula	Produced by
ω-Hydroxyemodin		P. cyclopium Westling[62a] P. citreo-roseum
Luteoleersin (C₂₈H₂₈O₇)		H leersii[59]
Monascoflavin (yellow) (C₁₇H₁₂O₄)		Monascus purpureus Went[62] (from old mycelium)
Monascorubrin (red) (C₂₂H₂₄O₅)		Monascus purpureus Went[62]
Ochracin		A. ochraceus[64]
Oosporin (purple-brown with FeCl₃) (C₁₆H₁₂O₃)		Oospora aurantia (Cooke) Sacc & Vogl[46]
Penetrinic acid (C₁₆H₁₇O₆N)		P. notatum[61c]
Penicilliopsin (C₁₆H₁₄O₄)		Penicilliopsis clavariaeformis[46b]
Phoenicin (red)		P. phoeniceum[68a, 77a] (old cultures) P. rubrum[78a]
Physcion		Species of A. glaucus[82] series
Ravenelin (3-Methyl-1:4:8-trihydroxyxanthone) (yellow) (C₁₄H₁₀O₅)		H ravenelii Curtis[40] H turcicum Passerini

Table 146 shows the structural formulas of several acids formed by molds, while on page 563 some of the acids are enumerated.

PIGMENTS

Several different types of coloring matter have been isolated from molds. Hydroxyanthraquinones are produced by species of *Helminthosporium*. For example, a mixture of helminthosporin and catenarin constituted about 30 per cent of the dried weight of the mycelium of *H. gramineum* Rabenhorst, there being 2 to 3 parts of the former substance to 1 part of the latter. Cynodontin and tritisporin are other hydroxyanthraquinones produced by species of *Helminthosporium*. Certain dyestuffs may be manufactured from α-hydroxyanthraquinones.

TABLE 148.—SOME MOLD POLYSACCHARIDES PRODUCED FROM GLUCOSE[1]

Polysaccharide	Products of hydrolysis	Produced by
Caprellinose	Galactose, glucose, malonic acid, and mannose	*Penicillium caprelinum*
Galactocarulose	d-Galactose	*P. charlesii* G. Smith
Glycogen	Glucose	A white species of *Aspergillus*
Luteic acid	Glucose and malonic acid	*P. luteum* Zukal
Mannocarulose	d-Mannose	*P. charlesii* G. Smith
Mold starch	Glucose	*A. fumigatus*, *A. glaucus*, *A. niger*, *A. oryzae*, *P. glaucum*, *P. variabile*
Mycodextrin	Glucose	*A. niger*, *P. expansum*
Rugulose	Galactose	*P. rugulosum*
Sclerutiose	Glucose	*P. sclerotiorum*
Varianose	d-Galactose, d-glucose, and either d-glose or l-altrose	*P. varians* G. Smith

[1] CLUTTERBUCK, P. W. *Jour. Soc. Chem. Ind.*, **53**, 557 (1936).

Thus it is within the realms of possibility that molds, utilizing glucose as the source of carbon, may sometime be used commercially in the production of dyestuffs.

Gould and Raistrick[1] isolated three crystalline pigments from species of the *Aspergillus glaucus* series. These included auroglaucin, an orange pigment; flavoglaucin, a yellow pigment; and rubroglaucin, a red pigment. Rubroglaucin has been shown to be a mixture of physcion, 4:5-dihydroxy-7-methoxy-2-methylanthraquinone, and erythroglaucin, a monomethyl ether of a tetrahydroxymethylanthraquinone.[2]

[1] GOULD, B. S., and H. RAISTRICK, *Biochem. Jour.*, **28**: 1640–1656 (1934).
[2] ASHLEY, J. N., H. RAISTRICK, and T. RICHARDS, *Biochem. Jour.*, **33**: 1291 (1939).

Ravenelin and rubrofusarin, two other pigments isolated from molds, are polyhydroxyxanthones

Some data concerning mold pigments are given in Table 147.

SOME MISCELLANEOUS PRODUCTS

Acetaldehyde.—Using fixation methods, acetaldehyde has been recovered from media fermented by species of the genera *Aspergillus, Monilia, Mucor, Oidium, Penicillium*, and other molds.

Ethyl Acetate.—This substance is produced by *P. digitatum* Sacc. from glucose media.

Ethyl Alcohol.—Although ethyl alcohol in small amounts is produced by species of several genera of molds, for example, *Aspergillus, Penicillium, Clasterosporium, Helminthosporium geniculatum*, and *Mucor racemosus*, it is produced commonly by species of the genus *Fusarium*. Yields of ethanol comparable to those obtained from yeasts have been produced by *F lini* Bolley [1]

The mechanism for the production of ethyl alcohol by fusaria has been studied by Anderson and his associates,[2] by White and Willaman,[3] by Nord,[4] by Gould, Tytell, and Hughes;[5] by Gould and Tytell,[6] and by others The mechanism for the production of ethyl alcohol and carbon dioxide by fusaria is possibly similar to that of yeasts, according to Gould and Tytell, who have shown the presence of cozymase and a carboxylase and cocarboxylase system similar to that in yeasts.

Glycerol.—Glycerol has been produced by a white species of *Aspergillus*, by *A ventii*, by a *Clasterosporium* species, by *H. geniculatum*, and by *M racemosus*.

Methylglyoxal.—Sodium hexosediphosphate has been fermented by *A. niger* with the production of methylglyoxal (Suthers and Walker, 1932).

Chlorine-containing Compounds.—Erdin $[C_{18}H_7O_5Cl_2(OCH_3)]$ and geodin $[C_{19}H_6O_5Cl_2(OCH_3)_2]$ are products of *A. terreus* Thom. Griseo-

[1] ANDERSON, A K, and J J WILLAMAN, *Proc. Soc Exptl Biol Med*, **20**: 108 (1922)

[2] PRITHAM, G H, and A K ANDERSON, *Jour Agr Research*, **55**: 937 (1937)

[3] WHITE, M G, and J J WILLAMAN, *Biochem Jour*, **22**: 592 (1928)

[4] NORD, F F, *Ergeb Enzymforsch*, **8**: 149–181 (1939) Abstracts of Communications, Third International Congress of Microbiology, New York, p 337, Sept 2–9, 1939

[5] GOULD, B S, A A TYTELL, and W L HUGHES, Jr, Abstracts of Communications, Third International Congress of Microbiology, p 51, New York, Sept 2–9, 1939

[6] GOULD, B S, and A A TYTELL. Unpublished data

fulvin $[C_{17}H_{17}O_6Cl]$ is a neutral, dextrorotatory crystalline substance produced by *P. griseo-fulvum* Dierckx.

Some Other Metabolic Products.—Table 149 supplies some information concerning fumigatin, mellein, spinulosin, and terrein.

MOLDS AND ARSENIC COMPOUNDS

Forty or more years ago the arsenic compounds present in the pigments of some old wallpapers and in some plosters were occasionally the cause of severe poisoning and even of fatalities. A fatal case of arsenic poisoning of this nature occurred in England as recently as 1931.[1]

A number of theories have been proposed to explain the cause of the poisoning. One of the earliest was that particles of the arsenic-containing pigment from the paper were inhaled. In 1839, Gmelin suggested that a volatile arsenic compound was responsible, since an odor of garlic was nearly always associated with the rooms where poisonings occurred. These rooms were commonly damp and moldy. Cacodyl oxide $[(CH_3)_2 \cdot As \cdot O \cdot As \cdot (CH_3)_2]$ was suggested by Basedow (1846) as being the substance concerned. One year later, Martin proposed that the gas might be arsine (AsH_3). The subject was carefully studied by Gosio (1891). Gosio exposed to the air potato mashes that contained arsenious oxide. Molds produced an odor of garlic from the potato mashes One mold, which was named *Penicillium brevicaule* by Gosio, was particularly active in this respect. Other molds concerned were *Aspergillus glaucus*, *A. virens*, and *Mucor mucedo*. The gas produced by these molds from arsenious oxide, frequently referred to as a "Gosio-gas," was shown by Challenger and his associates[2] to be trimethylarsino $[(CH_3)_3 \cdot As]$.

Thom and Raper[3] have shown that *A. fischeri*, *A. sydowi*, and other organisms isolated from the soil may produce volatile substances from arsenic compounds.

Challenger and his associates have shown that several organic compounds of arsenic may be formed by certain molds. For example, methyldiethylarsine is produced from diethylarsonic acid $[(CH_3CH_2)_2 \cdot AsO \cdot OH]$; dimethyl-n-propylarsine from n-propylarsonic acid; and dimethylallylarsine $[(CH_3)_2As \cdot CH_2 \cdot CH : CH_2]$ from allylarsonic acid $[CH_2 \cdot CH \cdot CH_2AsO(OH)_2]$ by the action of *P. brevicaule* Saccardo, grown on sterile bread crumbs.

Mixed alkylmethylarsines $[AsR(CH_3)_2$ and $AsR_2 \cdot CH_3]$ are obtained with alkyl-arsonic and dialkyl-arsonic acids $(R \cdot AsO_3H_2$ and $R_2AsO \cdot OH)$

[1] CHALLENGER, F, *Jour. Soc. Chem. Ind*, **54**: 657-662 (1935).
[2] CHALLENGER, F. C. HIGGINBOTTOM, and L. ELLIS, *Jour. Chem. Soc*, p. 95 (1933).
[3] THOM, C, and K. RAPER, *Science*, **76**: 548 (1932)

TABLE 149.—SOME MISCELLANEOUS MOLD METABOLIC PRODUCTS

Product	Structural formula	Produced by
Fumigatin (3-Hydroxy-4-methoxy-2:5-toluquinone) $(C_8H_8O_4)$		*Aspergillus fumigatus* Fresenius[47]*
Gentisyl alcohol (2 5-dihydroxy-benzyl alcohol)		*P. patulum* Bainier[44a]
Griseofulvin $(C_{17}H_{17}O_6Cl)$		*P. griseo-fulvum* Dierckx[56]
Mellein (A lactone of 6-hydroxy-2-α-hydroxypropylbenzoic acid) $(C_{10}H_{10}O_3)$		*A. melleus*[49] *A. ochraceus*
Spinulosin (3 6-Dihydroxy-4-methoxy 2 5-toluquinone) $(C_8H_8O_5)$		*Penicillium spinulosum* Thom[47 48 49]
Terrein (4-Propenyl-2-hydroxy-3 5-oxidocyclopentan-1-one) $(C_8H_{10}O_3)$		*A. terreus* Thom[53 54]

* The numbers in parentheses refer to the bibliography at the end of the chapter

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[1] CHALLENGER, F., *Jour. Soc. Chem. Ind*, **54**: 657–662 (1935).

[2] CHALLENGER, F., C. HIGGINBOTTOM, and L. ELLIS, *Jour. Chem. Soc.*, p. 95 (1933).

[3] THOM, C., and K. RAPER, *Science*, **76**: 548 (1932).

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* The numbers in parentheses refer to the bibliography at the end of the chapter

from bread inoculated with *P. brevicaule*. Dimethyl selenide is produced from sodium selenate and selenite.

For other examples of methylating action, consult the papers by Challenger and his coworkers.

Qualitative Test for Arsenic.—Gosio[1] developed an extremely sensitive test for the detection of arsenic. The material suspected of containing arsenic was extracted with water or dilute acid. The extract was concentrated by evaporation of some of the water. Some of the concentrated extract was then added to a slice of potato that had been previously sterilized and inoculated with a culture of *P. brevicaule*, and the medium was incubated at 25 to 30°C. An odor of garlic would develop if a compound of arsenic was present. This test is said to be more sensitive than the Marsh test and to be able to detect as small a quantity as 0 000,001 g. of arsenious oxide in 1 g. of substance.

References on the Biochemistry of Molds

I. Studies by Raistrick and His Associates[2]

1 Raistrick, H., and W. Rintoul: I. Introductory, p 2.

2. Birkinshaw, J. H., and H. Raistrick: II. Quantitative Methods and Technique of Investigation of the Products of Metabolism of Micro-organisms, p 11

3 ———, J. H. V Charles, H. Raistrick, and J. A. R. Stoyle: III. Quantitative Examination by the Carbon Balance Sheet Method of the Types of Products formed from Glucose by Species of *Aspergillus*, p. 27.

1 ———, ———, A C. Hetherington, and H. Raistrick: IV. Quantitative Examination by the Carbon Balance Sheet Method of the Types of Products formed from Glucose by Species of *Penicillium* (including *Citromyces*), with an appendix by C Thom, p 55.

5. ———, ———, H Raistrick, and J. A. R. Stoyle: V. Quantitative Examination by the Carbon Balance Sheet Method of the Types of Products formed from Glucose by Species of *Fusarium*, p. 93

6. ———, ———, A C. Hetherington, and H Raistrick: VI. Quantitative Examination by the Carbon Balance Sheet Method of the Types of Products formed from Glucose by Miscellaneous Species of Fungi, p. 99.

7. ———, ———, C. H Lilly, and H. Raistrick: VII. On Kojic Acid (5-Hydroxy-2-hydroxy-methyl-γ-pyrone), p 127.

8. ——— and H Raistrick: VIII. The Estimation of Kojic Acid, p. 139.

9 ———, J. H V Charles, A C Hetherington, and H Raistrick: IX. On the Production of Mannitol from Glucose by Species of *Aspergillus*, p 153.

10 Raistrick, H., and W. Young: X. The Estimation of Mannitol in Fermentation Solution, p. 173.

11. Hetherington, A. C., and H. Raistrick: XI. On Citromycetin, a New Yellow Colouring Matter Produced from Glucose by Species of *Citromyces*, p. 209.

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[1] Challenger, F., *Jour. Soc. Chem. Ind.*, **28**: 657–662 (1935).

[2] The first 18 of these studies were published in the *Philosophical Transactions of the Royal Society of London, Series B*, **220**: 1–367 (1931), under the title "Studies in the Biochemistry of Micro-organisms" by Prof. Harold Raistrick and his associates

12 BIRKINSHAW, J. H., and H RAISTRICK: XII. On a New Methoxy-dihydroxytoluquinone Produced from Glucose by Species of *Penicillium* of the *P. spinulosum* Series, p. 245.

13 RAISTRICK, H., and M. L RINTOUL: XIII. On a New Type of Mucilaginous Material, Luteic Acid, Produced from Glucose by *Penicillium luteum* Zukal, p 255.

14 HETHERINGTON, A. C., and H. RAISTRICK: XIV. On the Production and Chemical Constitution of a New Yellow Colouring Matter, Citrinin, Produced from Glucose by *Penicillium citrinum* Thom, p 269.

15 COYNE, F P., H. RAISTRICK, and R ROBINSON: XV. The Molecular Structure of Citrinin, p. 297.

16 CLUTTERBUCK, P W., H RAISTRICK, and M L RINTOUL: XVI On the Production from Glucose by *Penicillium spiculisporum* Lehman of a New Polybasic Fatty Acid, $C_{17}H_{28}O_6$ (the Lactone of γ-Hydroxy-$\beta\delta$-dicarboxy-pentadecoic acid), p 301.

17 BIRKINSHAW, J H , and H RAISTRICK. XVII The Products of Glucose Metabolism Formed by Various Species of Fungi (*Helminthosporium, Clasterosporium*, etc), p. 331.

18 ———, J. H. V CHARLES, and H RAISTRICK: XVIII. Biochemical Characteristics of Species of *Penicillium* Responsible for the Rot of Citrus Fruits, p 355

19 ANSLOW, W H , and R RAISTRICK XIX 6-Hydroxy-2-methylbenzoic Acid, a Product of the Metabolism of Glucose by *Penicillium griseo-fulvum* Dierckx, *Biochem Jour* , 25: 39 (1931).

20 COYNE, F P , and H RAISTRICK: XX On the Production of Mannitol from Hexoses and Pentoses by a White Species of *Aspergillus, Biochem Jour*, 25: 1513 (1931).

21 BIRKINSHAW, J. H , J H V CHARLES, and P W CLUTTERBUCK XXI Quantitative Examination by the Carbon Balance Sheet Method of the Types of Products Formed from Glucose by Species of Bacteria, *Biochem Jour* , 25: 1522 (1931)

22 ———, R K CALLOW, and C F FISCHMANN: XXII The Isolation and Characterisation of Ergosterol from *Penicillium puberulum* Bainier Grown on a Synthetic Medium with Glucose as a Sole Source of Carbon, *Biochem. Jour.*, 25: 1977 (1931).

23. ——— and H. RAISTRICK: XXIII Puberulic Acid $C_8H_6O_6$ and an Acid $C_8H_4O_6$, New Products of the Metabolism of Glucose by *Penicillium puberulum* Bainier and *Penicillium aurantio-virens* Biourge, with an Appendix on Certain Dihydroxybenzene-dicarboxylic Acids, *Biochem Jour* , 26: 441 (1932)

24 CLUTTERBUCK, P W , A E OXFORD, H RAISTRICK, and G SMITH XXIV The Metabolic Products of the *Penicillium brevi-compactum* Series, *Biochem. Jour.*, 26: 1441 (1932).

25 OXFORD, A. E , and H. RAISTRICK: XXV. 3·5-Dihydroxyphthalic Acid, a New Product of the Metabolism of Glucose by *Penicillium brevi-compactum* Dierckx and Related Species, *Biochem. Jour.*, 26: 1902 (1932)

26 CLUTTERBUCK, P W , R LOVELL, and H RAISTRICK XXVI The Formation from Glucose by Members of the *Penicillium chrysogenum* Series of a Pigment, an Alkali Soluble Protein, and Penicillin—the Antibacterial Substance of Fleming, *Biochem Jour.*, 26: 1907 (1932)

27 BIRKINSHAW, J H , and H RAISTRICK: XXVII. The Production of Luteic Acid from Various Sources of Carbon by *Penicillium luteum* Zukal, *Biochem. Jour.*, 27: 370 (1933).

28. CHARLES, J. H. V., H. RAISTRICK, R. ROBINSON, and A. R. TODD: XXVIII. Helminthosporin and Hydroxyisohelminthosporin, Metabolic Products of the Plant Pathogen *Helminthosporium gramineum* Rabenhorst, Biochem. Jour., 27: 499 (1933).

29. RAISTRICK, H., and P. SIMONART: XXIX. 2:5-Dihydroxybenzoic Acid (Gentisic Acid), a New Product of the Metabolism of Glucose by *Penicillium griseo-fulvum* Dierckx, Biochem. Jour., 27: 628 (1933).

30. OXFORD, A. E., and H. RAISTRICK: XXX. The Molecular Constitution of the Metabolic Products of *Penicillium brevi-compactum* Dierckx and Related Species. I. The Acids $C_{19}H_{16}O_6$, $C_{18}H_{16}O_6$, and $C_{18}H_{16}O_7$, Biochem. Jour., 27: 634 (1933).

31. CLUTTERBUCK, P. W., and H RAISTRICK: XXXI. The Molecular Constitution of the Metabolic Products of *Penicillium brevi-compactum* Dierckx and Related Species. II. Mycophenolic Acid, Biochem. Jour., 27: 654 (1933).

32. RAISTRICK, H., R ROBINSON, and A R TODD: XXXII. Cynodontin (1:4:5:8-Tetrahydroxy-2-Methylanthraquinone), a Metabolic Product of *Helminthosporium cynodontis* Marignoni and *Helminthosporium euchlaenae* Zimmermann, Biochem. Jour., 27: 1170 (1933).

33 OXFORD, A. E., and H. RAISTRICK: XXXIII. The Mycelial Constituents of *Penicillium brevi-compactum* Dierckx and Related Species. I. Ergosterol Palmitate, Biochem. Jour., 27: 1176 (1933).

34 ——— and ———: XXXIV A Note on the Mechanism of the Production of Phenolic Acids from Glucose by *Penicillium brevi-compactum* Dierckx, Biochem. Jour., 27: 1473 (1933).

35. RIASTRICK, H., and G SMITH: XXXV. The Metabolic Products of *Byssochlamys fulva* Olliver and Smith, Biochem. Jour., 27: 1814 (1933).

36 CLUTTERBUCK, P W., W N HAWORTH, H. RAISTRICK, G. SMITH, and M STACEY: XXXVI. The Metabolic Products of *Penicillium charlesi* G. Smith, Biochem. Jour., 28: 94 (1934).

37 RAISTRICK, H., R ROBINSON, and A. R TODD: XXXVII. (a) On the Production of Hydroxyanthraquinones by Species of *Helminthosporium*. (b) Isolation of Tritisporin, a New Metabolic Product of *Helminthosporium tritici-vulgaris* Nisikado. (c) The Molecular Constitution of Catenarin, Biochem. Jour., 28: 559 (1934).

38 BIRKINSHAW, J H., and H RAISTRICK: XXXVIII. The Metabolic Products of *Penicillium minio-luteum* Dierckx. Minioluteic Acid, Biochem. Jour., 28: 828 (1934)

39 OXFORD, A E, and H RAISTRICK: XXXIX The Metabolic Products of *Penicillium cratersforme* Gilman and Abbott, Biochem. Jour., 28: 1321 (1934)

40. GOULD, B S, and H. RAISTRICK: XL. The Crystalline Pigments of Species in the *Aspergillus glaucus* Series, Biochem. Jour., 28: 1640 (1934).

41. CLUTTERBUCK, P. W., H. RAISTRICK, and F. REUTER: XLI The Metabolic Products of *Penicillium charlesi* G. Smith. II. The Molecular Constitution of Carolic and Carolinic Acids, Biochem. Jour., 29: 300 (1935).

42. RAISTRICK, H., and G SMITH: XLII. The Metabolic Products of *Aspergillus terreus* Thom A New Mould Metabolic Product—Terrein, Biochem. Jour., 29: 606 (1935).

43. CLUTTERBUCK, P. W., H. RAISTRICK, and F. REUTER: XLIII. The Metabolic Products of *Penicillium charlesi* G. Smith. III. The Molecular Constitution of Carlic and Carlosic Acids, Biochem. Jour., 29: 871 (1935)

44. OXFORD, A. E., H. RAISTRICK, and P. SIMONART: XLIV. Fulvic Acid, a New Crystalline Yellow Pigment, a Metabolic Product of *P. griseo-fulvum* Dierckx, *P. flexuosum* Dale and *P. brefeldianum* Dodge, *Biochem. Jour.*, 29: 1102 (1935).

45. CLUTTERBUCK, P. W., H. RAISTRICK, and F. REUTER: XLV. The Metabolic Products of *Penicillium charlesii* G. Smith. IV. *l*-γ-Methyltetronic Acid, with Observations on the Formation and Structure of Ramigenic and Verticillic Acids, *Biochem. Jour.*, 29: 1300 (1935).

46. OXFORD, A. E., and H. RAISTRICK: XLVI. 1-Erythritol, a New Metabolic Product of *Penicillium brevi-compactum* Dierckx and *P. cyclopium* Westling, *Biochem. Jour.*, 29: 1599 (1935).

47. ANDERSON, C. G., and H. RAISTRICK: XLVII. The Production of Polysaccharides by *Penicillium luteum* Zukal, *Biochem. Jour.*, 30: 16 (1936).

48. BIRKINSHAW, J. H., A. E. OXFORD, and H. RAISTRICK: XLVIII. Penicillic Acid, a Metabolic Product of *Penicillium puberulum* Bainier and *P. cyclopium* Westling, *Biochem. Jour.*, 30: 394 (1936).

49. —— and H. RAISTRICK: XLIX. Palitantin, $C_{14}H_{22}O_4$, a Hitherto Undescribed Metabolic Product of *Penicillium palitans* Westling, *Biochem. Jour.*, 30: 801 (1936).

50. RAISTRICK, H., R. ROBINSON, and E. WHITE: L. Ravenelin (3-Methyl-1:4:8-Trihydroxyxanthone), a New Metabolic Product of *Helminthosporium ravenelii* Curtis and of *H. turcicum* Passerini, *Biochem. Jour.*, 30: 1303 (1936).

51. —— and G. SMITH. LI. The Metabolic Products of *Aspergillus terreus* Thom. II. Two New Chlorine-containing Mould Metabolic Products, Geodin and Erdin, *Biochem. Jour.*, 30: 1315 (1936).

52. BIRKINSHAW, J. H., and H. RAISTRICK. LII. Isolation, Properties and Constitution of Terrestric Acid (Ethylearolic Acid), a Metabolic Product of *Penicillium terrestre* Jensen, *Biochem. Jour.*, 30: 2194 (1936).

53. ASHLEY, J. N., B. C. HOBBS, and H. RAISTRICK: LIII. The Crystalline Colouring Matters of *Fusarium culmorum* (W. G. Smith) Saec. and Related Forms, *Biochem. Jour.*, 31: 385 (1937).

54. CLUTTERBUCK, P. W., H. RAISTRICK, and F. REUTER: LIV. The Molecular Constitution of Terrein, a Metabolic Product of *Aspergillus terreus* Thom. *Biochem. Jour.*, 31: 987 (1937).

55. ——, W. KOLBNER, and H. RAISTRICK. LV. The Molecular Constitution of Geodin and Erdin, Two Chlorine-containing Metabolic Products of *Aspergillus terreus* Thom. I. The Constitutional Relationship of Geodin and Erdin, *Biochem. Jour.*, 31: 1089 (1937).

56. ASHLEY, J. N., and H. RAISTRICK. LVI. Luteoleersin and Alboleersin, Metabolic Products of *Helminthosporium leersii* Atkinson. A Possible Oxidation-reduction System, *Biochem. Jour.*, 32: 449 (1938).

57. ANSLOW, W. K., and H. RAISTRICK: LVII. Fumigatin (3-Hydroxy-4-Methoxy-2,5-Toluquinone) and Spinulosin (3:6-Dihydroxy-4-Methoxy-2:5-Toluquinone) Metabolic Products Respectively of *Aspergillus fumigatus* Fresenius and *Penicillium spinulosum* Thom, *Biochem. Jour.*, 32: 687 (1938).

58. —— and ——. LVIII. Synthesis of Spinulosin (3:6-Dihydroxy-4-Methoxy-2:5-Toluquinone) a Metabolic Product of *Penicillium spinulosum* Thom, *Biochem. Jour.*, 32: 803 (1938).

59. RAISTRICK, H., and W. K. ANSLOW: LIX. Spinulosin (3:6-Dihydroxy-4-Methoxy-2,5-Toluquinone) a Metabolic Product of a Strain of *Aspergillus fumigatus* Fresenius, *Biochem. Jour.*, 32: 2288 (1938).

60. Oxford, A. E., H. Raistrick, and P. Simonart: LX. Griseofulvin $C_{17}H_{17}O_6Cl$, a
    Metabolic Product of *Penicillium griseo-fulvum* Dierckx, Biochem. Jour., 33:
    240 (1939).

61. Calam, C. T., P. W. .... : ....
    Molecular Con
    bolic Products o........ .... II. Dihydrogeodin and Dihydro-
    erdin Ethers, Biochem. Jour., 33: 579 (1939).

62. Ashley, J. N., H. Raistrick, and T. Richards: LXII. The Crystalline Colour-
    ing Matters of Species in the *Aspergillus glaucus* Series II, Biochem. Jour.,
    33: 1291 (1939).

63. Calam, C. T., A. E. Oxford, and H. Raistrick: LXIII. Itaconic Acid, a
    Metabolic Product of a Strain of *Aspergillus terreus* Thom, Biochem. Jour., 33:
    1488 (1939).

63a. Anslow, W. K., J. Breen, and H. Raistrick: 64. Emodic Acid (4:5:7-Tri-
    hydroxyanthraquinone-2-carboxylic Acid) and ω-Hydroxy-Emodin (4:5:7-
    T........ ....   "   Products of a
    .... "   ....   ..   , I (No. 2): 153-165
    (1910).

63b. Clutterbuck, P. W., S. L. Mukhopadhyay, A. E. Oxford, and H. Raistrick:
    65 (A) A Survey of Chlorine Metabolism by Moulds. (B) Caldariomycin,
    $C_6H_8O_2Cl_2$, A Metabolic Product of *Caldariomyces fumago* Woronichin, Biochem.
    Jour., 34 (No. 5): 664-677 (1910).

63c. Oxford, A. E., and H. Raistrick: 66. Peniciliiopsin, the Colouring Matter of
    Peniciliopsis clavariaeformis Solms-Laubach, Biochem. Jour., 34 (No. 6):
    790-803 (1940).

63d. Anslow, W. K., and H. Raistrick: 67. The Molecular Constitutions of Catena-
    rin and Erythroglaucin, Metabolic Products Respectively of *Helminthosporium
    catenarium* Drechsler and of Species in the *Aspergillus glaucus* series, Biochem.
    Jour., 34 (No. 7): 1124-1133 (1940)

63e. ———— and ————: 68 Synthesis of Cynodontin (1:4:5:8-tetrahydroxy-2-
    methylanthraquinone), A Metabolic Product of Species of *Helminthosporium*,
    Biochem Jour., 34 (No. 12): 1516-1518 (1940)

63f. ———— and ————: 69. Synthesis of Catenarin (1:4:5·7-tetrahydroxy-2-
    methylanthraquinone), a Metabolic Product of Species of *Helminthosporium*,
    Biochem Jour., 35 (Nos 8 & 9): 1006-1010 (1941)

63g. Birkinshaw, J H, A R. Chambers, and H. Raistrick. 70 Stipitatic Acid,
    $C_8H_6O_4$, a Metabolic Product of *Penicillium stipitatum* Thom, Biochem. Jour.,
    36 (Nos 1 & 2) 242-251 (1942).

63h. ————, H Raistrick, and G Smith: 71. Fumaryl-dl-alanine (Fumaromono-
    dl-alanide), a Metabolic Product of *Penicillium resticulosum* sp. nov, Biochem.
    Jour., 36 (Nos 10, 11, & 12): 829-835 (1942).

63i. ————, A. Bracken, and H Raistrick: 72 Gentisyl Alcohol (2:5-dihydroxy-
    benzyl alcohol), a Metabolic Product of *Penicillium patulum* Banner, Biochem.
    Jour., 37 (No. 6): 726-728 (1943).

63j. ————, ————, and ————: 73 Metabolic Products of *Aspergillus fumigatus*
    Fresenius, Biochem Jour., 39 (No 1). 70-72 (1945).

64. Haworth, W. N., H. Raistrick, and M Stacey: Polysaccharides Synthesized
    by Micro-organisms. I. The Molecular Structure of Mannocarolose Produced
    from Glucose by *Penicillium charlesii* G. Smith Biochem. Jour., 29: 612 (1935)

65. ————, ————, and ————: Polysaccharides Synthesized by Micro-organisms

II. The Molecular Structure of Varianose Produced from Glucose by Penicillium varians G Smith, Biochem. Jour., 29: 2668 (1935).

66. ANDERSON, G. G., W N. HAWORTH, H RAISTRICK, and M. STACEY: Polysaccharides Synthesized by Micro-organisms IV The Molecular Constitution of Luteose, Biochem Jour, 33: 272 (1939).

II GENERAL STUDIES

66a ALBERICCI, V J., T. P. CURTIN, and D REILLY Sclerotiose- a Polysaccharide Metabolic Product of Penicillium sclerotiorum Van Beyma, Biochem Jour., 37: 243-246 (1943)

67 ANDERSON, A K, E L EVERETT, and P D ADAMS: The Carbon Metabolism of Fusarium oxysporum on Glucose, Jour Agr Research, 46: 473 (1933).

68 ——— and J J WILLAMAN Proc Soc Exptl Biol Med., 20: 108 (1922).

69 BIRKINSHAW, J H Biochemistry of the Lower Fungi, Biol. Rev, Cambridge Phil Soc, 12: 357 (1937)

70 CHALLENGER, F.: The Biological Methylation of Compounds of Arsenic and Selenium, Jour Soc Chem Ind, 54: 657-662 (1935)

71 ——— and L ELLIS: The Formation of Organo-metalloidal Compounds by Micro-organisms Part III Methylated Alkyl- and Dialkyl-arsines, Jour Chem Soc, 87: 396-400 (1935)

72. ——— G HIGGINBOTTOM, and L ELLIS The Formation of Organo-metalloidal Compounds by Micro-organisms Part I Trimethylarsine and Dimethyl-ethylarsine, Jour Chem Soc, P 95 (1933)

73 ——— and L KLEIN The Formation of l-Malic Acid from Fumaric Acid by Aspergillus niger, Jour Chem Soc, P 1644 (1929)

74 ——— and A A RAWLINGS The Formation of Organo-metalloidal Compounds by Micro-organisms Part IV Dimethyl-n-propylarsine and Methylethyl-n-propylarsine, Jour Chem Soc, 87: 264-267 (1936)

75 CLUTTERBUCK, P W · Recent Developments in the Biochemistry of Moulds, Jour Soc Chem Ind, 55: 55T (1936)

75a CURTIN, T, G, FITZGERALD, and J REILLY: Production of Phoenicine on Synthetic Media 1 Penicillium phoeniceum Van Beyma 2 Penicillium rubrum Grasberger-Stoll, Biochem Jour., 34: 1605-1610 (1940)

75b. ——— and J REILLY Sclerotiorine, C$_{21}$H$_{23}$O$_5$Cl, A Chlorine-Containing Metabolic Product of Penicillium sclerotiorum Van Beyma, Biochem Jour., 34: 1419-1421 (1940)

76 DOX, A W, and R E NEIDIG The Soluble Polysaccharides of Lower Fungi I Mycodextrin, a New Polysaccharide in Penicillium expansion, Jour Biol Chem, 18. 167 (1914)

77 FREIDHEIM, E A H Sur la fonction respiratoire du pigment rouge de Penicillium phoeniceum, Compt rend soc biol, 112: 1030 (1933)

77a FREIDHEIM, E A H Recherches sur la biochimie des champignons inférieurs I Isolement du pigment rouge de Penicillium phoeniceum (Phoenicine), Helv Chim Acta, 21: 1464-1465 (1938)

77b GOULD, B S Chemical Compounds Formed from Sugars by Molds, Scientific Report Series No 7, Sugar Research Foundation, Inc . New York June 1917

78 ———, A A TYTELL, W L HUGHES, Jr Studies in the Biochemistry of the Fusaria, Abstracts of Communications, Third International Congress of Microbiology, pp 51-52, New York, Sept 2-9, 1939

79 ——— and A A TYTELL Unpublished data

79a. HIND, H. G.: The Colouring Matters of *Penicillium carmino-violaceum* Bourge, with a Note on the Production of Ergosterol by This Mold, *Biochem. Jour.*, 34: 67–72 (1940).

79b. ———: The Constitution of Carviolin: A Colouring Matter of *Penicillium carmino-violaceum* Biourge *Biochem. Jour.*, 34: 577–579 (1940).

79c. IGARASI, H.: *Jour. Chem. Soc. (Japan)*, 15: 229 (1939).

80 IWANOFF, N. N., and E. S. ZWETKOFF: The Biochemistry of the Fungi, *Ann Rev Biochem.*, 5: 585 (1936).

81. KINOSHITA, K.: Über die produktion von Itaconsäure und Mannit durch einen neuen Schimmelpilze, *Aspergillus itaconicus*, *Acta Phytochim. (Japan)*, 5: 271 (1932).

82. KÖGL, F., und W. B. DEISS: Untersuchungen über Pilzfarbstoffe. XI. Über Boletol, den Farbstoff der blau anlaufener Boleten, *Ann.*, 515: 10 (1934).

83. LINOSSIER, G.: Sur une hématine végétale, l'aspergilline, *Compt rend.*, 112: 807 (1891).

84. LOCKWOOD, L. B., and A. J. MOYER: The Production of Chemicals by Filamentous Fungi, *Botan. Rev.*, 4: 140 (1938).

85. MAY, O. E., and G. E. WARD: Hydrolysis of the Chitinous Complex of Lower Fungi, *Jour. Amer. Chem Soc.*, 56: 1597 (1934).

85a. MULL, R. P., and F. F. NORD: Structure and Action of Rubrofusarin from *Fusarium graminearum* Schwabe (Tgrs) (*Gibberella saubinetii*), *Arch. Biochem.*, 4: 419–433 (1944).

86 NORD, F. F.: Enzymatische Umsetzungen durch Fusarien: Beitrag zum Mechanismus der alkoholischen   ⌐ ... ... (1939)

87. ———: On the Mechanism   ...uni-
     cations, Third Internatio....   337,
     Sept. 2–9, 1939

88. NORMAN, A. G., and W. H. PETERSON: The Chemistry of Mold Tissue   II   The Resistant Cell Wall Material, *Biochem. Jour*, 26: 1946 (1932).

88a. P................, T.: Recherches sur la biochimie des champignons inférieur II. Sur
                                                                                     ...ie 6..

89. PRITHAM, G H., and A H ANDERSON: The Carbon Metabolism of ..lycopersici on Glucose, *Jour Agr Research*, 55: 937 (1937).

90 PRUESS, L. M., W H. PETERSON, H. STEENBOCK, and E. B. FRED: Sterol Content and Antirachitic Activatability of Mold Mycelia, *Jour. Biol. Chem.*, 90: 369 (1931).

91. RAISTRICK, H.: Certain Aspects of the Biochemistry of the Lower Fungi ("Moulds"), *Ergeb Enzymforsch*, 7: 316–349 (1938).

91a ———: Biochemistry of the Lower Fungi, *Ann. Rev. Biochem*, 9: 571–592 (1940).

91b. SAKAGUCHI, K, T. INOUE, and S TADA: *Zentr. Bakt Parasitenk II.*, 100: 302 (1939).

91c STODOLA, F H, J L. WACHTEL, A J. MOYER, and R D. COGHILL: Penitranic Acid, a New Pigment from *Penicillium notatum*, *Jour. Biol. Chem*, 159 (No 1) 67–70 (1945).

92 SUMIKI, Y.: Studies on the Fermentation Products of Mould Fungi. X. Glauce Acid Formed by *Aspergillus glaucus* (*Aspergillus glaucus* IV), *Bull. Agr. Chem Soc. (Japan)*, 9: 105 (1933).

92a. TATUM, E L.: Biochemistry of Fungi, *Ann. Rev. Biochem*, 13: 667–704 (1944).

93. Thom, C., and K. Rafer: The Arsenic Fungi of Gosio, *Science*, **75**: 548 (1932).
94. Wenck, P. R., W. H. Peterson, and E B Fred: The Chemistry of Mold Tissue. IX. Cultural Factors Influencing the Growth and Sterol Production by *Aspergillus fischeri*, *Centr Bakt. Parasitenk*, Abt II, **92**: 330 (1935).
95. ———, ———, and H. C Greene: The Chemistry of Mold Tissue VIII Innate Factors Influencing Growth and Sterol Production of *Aspergillus fischeri*, *Centr. Bakt. Parasitenk.*, Abt. II, **92**: 324 (1935)
96. White, M. G., and J. J. Willaman: *Fusarium lini* and the Pyruvic Acid Theory of Alcoholic Fermentation, *Biochem Jour*, **22**: 592 (1928)
97. Woolley, D. W., and W. H Peterson: The Chemistry of Mold Tissue. XI. Isolation of Leucine and Isoleucine from *Aspergillus sydowi*, *Jour Biol Chem*, **114**: 85 (1936).
98. Wunschendorff, M, et C Killian, Observations sur le métabolisme de l'*Ustulina vulgaris* L. *Compt rend*, **187**: 572 (1928).

ANTIBIOTICS

An antibiotic is a chemical substance produced by a living organism that demonstrates inhibitory or germicidal activity towards microorganisms *in vivo* and/or *in vitro*. The term antibacterial is sometimes used instead of antibiotic to designate a substance active against bacteria.

Antibiotic substances are produced by certain members of the plant kingdom, chiefly by microorganisms and green plants. Most of the antibiotics isolated and studied up to the present time have been produced by fungi and bacteria. The most important sources of antibiotics among the fungi are the penicillin, actinomycetes, aspergilli, and higher fungi. The two most important antibiotics isolated to date, the penicillins and streptomycin, are produced by penicillia and an actinomycete, respectively. Antibiotics of considerable promise, such as bacitracin, have been isolated from bacteria. Tomato plants, horse chestnuts, radish seeds, and other plants are sources of antibacterial substances.

There have been many reviews concerning the antibiotics Among some of the more recent general reviews may be mentioned those of Waksman,[1] Benedict and Langlykke,[2] Kavanagh,[3] and Nadel.[4]

Some general information concerning antibiotics of microbial origin is presented in Tables 150, 151, and 152. In Table 153 are given the structural formulas for some of these antibiotics.

Factors Considered in Selection of Antibiotic.—After perusal of Tables 150 to 152 one may wonder why there are not a larger number of antibiotics being manufactured for use in the medical field than there are at the present time. The reason is that the requirements of the antibiotic for use in treating man are high. For example, the antibiotic must be active against the pathogen *in vivo* Some antibiotic substances are active only *in vitro* or to a very limited extent *in vivo*. The antibiotic should be active against a fairly large number of microorganisms, although this is not always essential. Some are actually effective against only a

[1] Waksman, S. A., "Microbial Antagonisms and Antibiotic Substances," 2d ed., Commonwealth Fund, New York, 1947.

[2] Benedict, R. G., and A. F Langlykke, *Ann. Rev. Microbiol*, 1: 193–236 (1947).

[3] Kavanagh, F , *Advances in Enzymol* , 7: 461–511 (1947)

[4] Nadel, R. G., "Antibiotic Substances, Their Biological and Chemical Properties," 2d ed., National Institute of Health, Bethesda, Md., February, 1948.

TABLE 150 —SOME ANTIBIOTIC SUBSTANCES OF BACTERIAL ORIGIN

Antibiotic	Produced by	Solubilities	Organisms against which active
Aerosporin * (Polymyxin?)	*Bacillus aerosporus (polymyxa)*		Gram-negative bacteria
Bacillin	*B subtilis*	Soluble in water, and in alcohol and methanol with 5 per cent water	Gram-positive and Gram-negative bacteria Not effective in vitro
Bacitracin	*B subtilis*	Soluble in water, 95 per cent alcohol, methyl alcohol, ethylene glycol, etc	Gram-positive bacteria Hemolytic streptococcus infections experimentally produced
Colicine	*Escherichia coli*	Soluble in water Insoluble in most organic solvents	Entero-bacteria
Diplococcin	Milk streptococci	Soluble in water Insoluble in absolute alcohol	Gram-positive bacteria
Endo-subtilin	*B. subtilis* (young cells)	Soluble in alcohol, chloroform	Staphylococci, etc.
Eumycin	*B subtilis*	Soluble in water, acetone, alcohol Insoluble in ether	Higher bacteria and fungi C diphtheriae and M tuberculosis in vitro
Gramicidin	*B brevis*	Soluble in alcohol and ether Insoluble in water	Gram-positive cocci in vivo and in vitro
Gramicidin-S	*B brevis var Gause-Brazhnikova*	Soluble in chloroform	Staphylococci, clostridia, etc
Iodinin	*Chromobacterium iodinum*	Soluble in ether	Staphylococci, streptococci
Licheniformin	*Bacillus licheniformis*		Certain Gram-positive and acid-fast bacteria
Oxyphenazine	*Pseudomonas aeruginosa*		Gram-positive and some Gram-negative bacteria
Phthiocol	*Mycobacterium tuberculosis*		Certain Gram-positive and Gram-negative bacteria
Polymyxin	*Bacillus polymyxa*	Soluble in water and methanol Insoluble in acetone, ether, etc.	Gram-negative bacteria
Prodigiosin	*Serratia marcescens*	Soluble in water	Gram-positive bacteria

TABLE 150.—SOME ANTIBIOTIC SUBSTANCES OF BACTERIAL ORIGIN.—(Continued)

Antibiotic	Produced by	Solubilities	Organisms against which active
Pyo II . .	*Pseudomonas aeruginosa*	Soluble in hot alcohol, benzene, chloroform, dioxane	Gram-positive bacteria
Pyocyanase. .	*Ps. aeruginosa*	Soluble in water, benzene, benzol, ether, etc.	Gram-positive and Gram-negative bacteria *in vitro*
Pyocyanin.	*Ps. aeruginosa*	Soluble in water and chloroform Insoluble in ether	Gram-positive bacteria and some Gram-negative bacteria *in vitro*
Pyolipic acid	*Ps. aeruginosa*		*M. tuberculosis*
Simplexin	*Bacillus simplex*	Soluble in water and hot alcohol	Pathogenic fungi and certain Gram-negative bacteria
Subtilin	*B. subtilis*	Soluble in acetic acid, alcohol (0 to 80 per cent), methanol and acidified water. Insoluble in acetone, amyl alcohol, chloroform and ether	Gram-positive bacteria, higher fungi, *M. tuberculosis*. Experimental infections *in vivo*
Subtilysin (subtilyne)	*B subtilis*		Certain Gram-negative and Gram-positive bacteria
Toxoflavin	*B cocovenenans*	Soluble in water and chloroform	
Trypanotoxin	*B subtilis*		Trypanosomes
Tyrocidine	*B brevis*	Soluble in alcohol Slightly soluble in water, acetone and dioxane Insoluble in acetone and ether	Gram-positive and Gram-negative bacteria
Tyrothricin (mixture of Gramicidin and tyrocidine)	*B brevis*	Insoluble in water, chloroform Soluble in alcohol	Gram-positive cocci
Violacein	*Chromobacterium violaceum*	Soluble in acetone and pyridine, and slightly so in alcohol	Gram-positive bacteria *in vitro*

TABLE 151.—SOME ANTIBIOTIC SUBSTANCES FROM ACTINOMYCETES[1]

Antibiotic	Produced by	Solubilities	Organisms against which active
Actidione	*Streptomyces griseus*	Soluble in water, acetone, chloroform, ether	Some yeasts
Actinomyces lysozyme	Strept sp	Insoluble in benzol, chloroform, ether	Micrococci
Actinomycetin	Strept albus	Soluble in water Precipitated by acetone, alcohol	Some Gram-positive living bacteria and Gram-negative dead bacteria
Actinomycin	Strept antibioticus	Soluble in acetone, alcohol and benzene. Slightly soluble in water and ether Insoluble in petroleum ether	Gram-positive bacteria and to some extent to Gram-negative bacteria, fungi
Actinorubin	*Actinomyces* strain A 105	Soluble in methanol Precipitated by ether	Gram-negative and Gram-positive bacteria *in vitro*
Aureomycin	*Streptomyces aureofaciens*	Soluble in distilled water and in 5 per cent glucose in distilled water	Some rickettsial and virus diseases Gram-positive and Gram-negative bacteria
Chloromycetin	*Streptomyces* sp	Slightly soluble in water, soluble in acetone, butanol, ethanol, methanol, etc	Some Gram-negative bacteria
Grisein	Some strains of *Streptomyces*	Soluble in water Slightly soluble in acetone and 95 per cent ethanol Insoluble in absolute acetone, benzene, absolute ethanol, chloroform, ether	Some Gram-positive and Gram-negative bacteria *in vitro* Experimental infections due to *Salmonella schottmulleri* and *Staph aureus*
Lavendulin	*Actinomyces lavendulae* (strain A-10)	Soluble in water, and methanol Insoluble in ether	Some Gram-positive and Gram-negative bacteria *in vitro* *Klebsiella pneumoniae* in experimentally infected mice
Litmocidin	*Proactinomyces cyaneus antibioticus*	Slightly soluble in acid range Soluble in acetone and ethanol	Staphylococci, streptococci, *Vibrio comma*, *M tuberculosis*

[1] A subdivision of the fungi.

TABLE 151.—SOME ANTIBIOTIC SUBSTANCES FROM ACTINOMYCETES.[1]—(Continued)

Antibiotic	Produced by	Solubilities	Organisms against which active
Mycetin	*Streptomyces violaceus*	Insoluble in ether, but soluble in other organic solvents	Staphylococci and other Gram-positive bacteria
Nocardine . .	*Nocardia coeliaca*	Soluble in water, alcohol	*M. tuberculosis in vitro*
Proactinomycein	*Nocardia gardneri*	Soluble in ether and other solvents of organic nature	Gram-positive bacteria and some Gram-negative bacteria *in vitro*. Hemolytic streptococci in experimentally infected mice
Streptin	*Streptomyces sp*		Staphylococci and micrococci, *M. tuberculosis*, etc.
Streptomycin .	*Strept griseus*	Soluble in water, acid alcohol, and methanol. Insoluble in butanol, ethanol and pyridine	Gram-negative bacteria, and some Gram-positive bacteria (refer to section on this antibiotic). Effective *in vivo*
Streptomycin B	*Strept. griseus*		Gram-negative and some Gram-positive bacteria (refer to later section)
Streptomycin II	*Strept. bikiniensis*		(Refer to Streptomycin)
Streptothricin	*Strept lavendulae*	Soluble in water and acid alcohol. Insoluble in ether and in other solvents of organic nature	Fungi, Gram-positive bacteria. Effective *in vivo*
Sulfactin	*Actinomyces B 30*	Limited solubility in water, benzene, ether. Soluble in butanol, chloroform, dioxane, ethanol, and ethyl acetate	Some Gram-positive bacteria

[1] A subdivision of the fungi

relatively few organisms, against which more suitable antibiotics are also effective. The antibiotic should not produce undesirable reactions upon administration, such as those associated with histamine or histamine-like substances. It should be relatively nontoxic to phagocytes and to the cells, tissues, and organs of the body. It should not hemolyze blood or

TABLE 152—SOME ANTIBIOTIC SUBSTANCES OF FUNGUS ORIGIN

Antibiotic	Produced by	Solubilities	Organisms against which active
Aspergillic acid	*Aspergillus flavus*	Nearly insoluble in water and acids Soluble in acetone, alcohol, benzene, ether etc.	Staphylococci, streptococci, *M tuberculosis*, etc , *in vitro*
Aspergillin	*A fumigatus*		
Avenacein....	*Fusarium avenaceum*	Soluble in organic solvents Limited solubility in water	*M phlei*
Biformic acid...	*Polyporus biformis*	Soluble in water, ether, acidified chloroform	*B subtilis, Staph aureus, M phlei, Ps. aeruginosa,* etc
Biformin.........	*P. biformis*	Moderately soluble in water Soluble in alcohol, chloroform, ether, methylisobutyl ketone	Gram-positive and Gram-negative bacteria, mycobacteria, fungi
Chaetomin... ..	*Chaetomium cochliodes*	Insoluble in water and petroleum ether Soluble in acetone, benzene, chloroform and ethyl acetate	Gram-positive bacteria Inactive *in vitro*
Citrinin...... .	*Penicillium citrinum, Aspergillus niveus,* etc	Insoluble in water Soluble in alcohol	Inhibits Gram-positive bacteria *in vitro*
Clavacin (clavatin, clavaformin, expansin, patulin)	*Penicillium patulum P claviforme P. expansum Aspergillus clavatus*	Soluble in water, alcohol, chloroform and ether	Gram-positive and Gram-negative organisms
Enniatin	*Fusarium orthoceras var enniatinum*	Insoluble in water	Mycobacteria, *B subtilis, Staph aureus,* in vitro
Fructigenin	*F fructigenum*	Limited solubility in water Very soluble in organic solvents	*M phlei*
Fumigacin	*Aspergillus fumigatus*	Slightly soluble in water Soluble in acetone, alcohol, chloroform, and ether	Gram-positive bacteria and some Gram-negative bacteria
Fumigatin	*A fumigatus* Fresinius	Moderately soluble in water. Soluble in acetone, alcohol, benzene, chloroform, ether, ethyl acetate	Gram-positive bacteria

TABLE 152—SOME ANTIBIOTIC SUBSTANCES OF FUNGUS ORIGIN.—(Continued)

Antibiotic	Produced by	Solubilities	Organisms against which active
Geodin	*A. terreus*	Soluble in aqueous acetone and in methanol	Gram-positive bacteria chiefly
Gigantic acid	*A. gigantus*	Refer to penicillin	Refer to penicillin
Gladiolic acid	*Penicillium gladioli*	Soluble in water and ether	Inhibits fungi
Gliotoxin . . .	*Aspergillus fumigatus* *Penicillium jenseni* *P. obscurum* Bi-ourgo *Gliocladium fimbriatum*	Almost insoluble in water. Soluble in alcohol, chloroform, ethyl acetate, etc.	Inhibits Gram-positive bacteria and fungi
Glutinosin .	*Metarrhizium glutinosum*	Soluble in n-butyl alcohol, ether, petroleum ether	Inhibits some fungi
Javanicin	*Fusarium javanicum*	Soluble in water, benzene and ether	Inhibits Gram-positive bacteria *in vitro*
Kojic acid	*Aspergillus flavus* *A. oryzae*, etc.	Soluble in water and in alcohol	Inhibits Gram-negative bacteria particularly but also Gram-positive bacteria
Lateritiin-I .	*Fusarium lateritium*	Limited solubility in water. Soluble in organic solvents	Inhibits *M. phlei* and *M. tuberculosis*
Mycophenolic acid	*Penicillium brevicompactum*	Insoluble in cold water	Inhibits staphylococci and certain fungi
Penicidin	*P. terrestre*	Soluble in alcohol, chloroform, and dilute acid. Insoluble in petroleum	Inhibits *E. typhosa*
Penicillic acid	*P. puberulum*		Inhibits bacteria of the colon-typhoid group
Penicillin	*P. notatum-chrysogenum* group	Soluble in water, acetone, alcohol, amyl acetate, ethyl acetate, cyclohexane, and dioxane	Gram-positive bacteria *in vivo* and *in vitro*
Polyporin . .	*Polystictus sanguineus*	Soluble in water, absolute alcohol, and ethyl acetate, fairly soluble in dry ether	Inhibits some Gram-positive and Gram-negative organisms

ANTIBIOTICS

TABLE 152 —SOME ANTIBIOTIC SUBSTANCES OF FUNGUS ORIGIN —(Continued)

Antibiotic	Produced by	Solubilities	Organisms against which active
Puberulic acid	Penicillium puberulum		Inhibits Gram-positive organisms chiefly
Puberulonic acid	P aurantiovirens		Inhibits Gram-positive and Gram-negative bacteria
Sambucinin	Fusarium sambucinum	Limited solubility in water Soluble in organic solvents	Inhibits M phlei
Spinulosin	Penicillium spinulosum Aspergillus fumigatus		Inhibits Gram-positive bacteria chiefly
Tardin	Penicillium tardum	Limited solubility in water Soluble in acetone, alcohol, amyl acetate, benzene and ether	Inhibits C diphtheriae grevis, Salmonella enteritidis, etc
Ustin	Aspergillus ustus		Inhibits Gram-positive cocci and mycobacteria
Viridin	Trichoderma viride	Soluble in hot ethyl and methyl alcohols, and in chloroform Insoluble in ether	Inhibits fungi

precipitate serum proteins It should be soluble in water or normal saline. It should be relatively stable Only a very few antibiotics satisfy most of these requirements, among them the penicillins, streptomycin, aureomycin, chloromycetin and bacitracin Others, such as tyrothricin, have limited applications and are manufactured on a small scale

Search for New Antibiotics.—The search for new antibiotics is being carried out in laboratories in many different countries with the hope of finding some that will have new uses in the treatment of diseases or infections for which cures are inadequate, poor, or lacking at the present time or of finding some that may be even better than penicillin and streptomycin
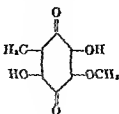
The discovery of an antibiotic substance may be the result of an opportune observation (usually rare) or the culmination of a carefully planned program of research wherein systematic surveys, studies, and analyses are carried out.

The researcher may examine all kinds of microorganisms, confine his

TABLE 153.—STRUCTURAL FORMULAS OF SOME ANTIBIOTIC SUBSTANCES

Antibiotic	Structural formula	Reference
Aspergillic acid ($C_{12}H_{20}N_2O_2$)		Dutcher, J. D., and O Wintersteiner, *Jour. Biol Chem.*, **155**: 359 (1944).
Citrinin ($C_{13}H_{14}O_5$)		Hetherington, A. C., and H. Raistrick, *Trans Roy Soc. (London)*, B220: 269 (1931)
Clavacin (clavatin, clavaformin, expansin, patulin) ($C_7H_6O_4$)		Anslow, W. K., H. Raistrick, and G. Smith, *Jour. Soc. Chem. Ind*, **62**: 236 (1943); Bergel, F, *et al* *Nature*, 152: 750 (1943); Waksman, S. A., E S Horning, and E. L. Spencer, *Science*, **96**: 202 (1942), etc.
Fumigatin ($C_8H_8O_4$)		Anslow, W. K, and H Raistrick, *Biochem. Jour.*, **32**: 687 (1938).
Kojic acid ($C_6H_6O_4$)		

TABLE 153 —STRUCTURAL FORMULAS OF SOME ANTIBIOTIC SUBSTANCES.—*(Continued)*

Antibiotic	Structural formula	Reference
Spinulosin ($C_8H_8O_5$)		Birkinshaw, J H , and H. Raistrick, *Trans. Roy Soc (London)*, B220: 245 (1931), Anslow, W K , and H. Raistrick, *Biochem Jour*, 32: 687 (1938)

search to the bacteria, actinomycetes, molds, or higher fungi, or limit his investigations to the species of a given genus or the strains of a particular species.

Large numbers of cultures are essential for such research. Some of these may be obtained from pure culture collections, but many must be isolated from such sources as the soil, water, air, surfaces of fruits and vegetables, moldy bread, and cheeses.

Some Isolation Methods.—No attempt will be made here to review the somewhat voluminous literature on this subject. The interested reader is referred to Waksman's book, "Microbial Antagonisms and Antagonistic Substances," for a general review of this field

In a procedure known as the crowded-plate method,[1] 1-ml portions of 1:10 to 1:1,000 dilutions of soil are plated with nutrient agar and incubated at temperature ranges suitable for the growth of bacteria Antagonistic organisms (those producing antibiotic substances) will produce clear zones around their colonies as a result of inhibiting the growth of adjacent bacteria or of destroying them. Such organisms are isolated in pure culture and the antibiotic is extracted, studied, and identified.

In a direct-soil-inoculation method,[2] plates are poured with nutrient agar which has been seeded with the organism for which an antagonist is desired The plates are incubated at 28 or 37°C. for 1 to 2 days. Then particles of soil (fresh or enriched) are dropped onto the surface of the growth on the plate, which is then incubated for a further period. An antagonistic organism will inhibit, destroy, or cause lysis of the original organism. Potato agar may be used instead of nutrient agar when it is desired to isolate an organism antagonistic to fungi, according to Waksman.[2]

Occasionally the researcher is interested in isolating new strains of a

[1] WAKSMAN, *op cit.*
[2] *Ibid.*

particular species or of a group of organisms for the purpose of obtaining a strain that is a better producer of a given antibiotic than known strains. Raper, Alexander, and Coghill[1] desired to isolate new strains of the *Penicillium notatum-chrysogenum* group and proceeded in the following general manner: The isolations were made by dilution and streak culture methods. In the former method, suitable dilutions of soil samples, usually 1:1,000 to 1:1,000,000, were plated in Czapek's medium or in an acid glucose nitrate agar. The plates were incubated for 5 to 10 days at 24 to 26°C. and then examined for molds displaying the desired characteristics of the penicillin-producing strains of the *P. notatum-chrysogenum* group. The streak method was carried out by streaking the surface of hay infusion agar with 1 to 4 loopfuls of a 1:10 suspension of soil, incubating the plates at 24 to 26°C. for 7 to 10 days, and examining the plates to find the desired species. Final isolations of molds grown on the hay infusion agar were made in Czapek's medium.

The molds that appeared to be members of the *P. notatum-chrysogenum* group were next planted on the surface of Czapek's solution agar in petri dishes by Raper and his associates, an attempt being made to produce two to four colonies, equally spaced, in each plate. The plates were incubated at room temperature for 10 to 12 days Then the penicillus, conidia, and other characteristics of each mold were examined to determine whether it possessed the desired qualifications. The appearance of an abundant yellow exudate was one of the distinguishing features

Provided that the mold turned out to be a member of the *P. notatum-chrysogenum* group, it was next submitted to a screening test to obtain some idea as to its value as a penicillin producer.

Raper and his coworkers used a medium designated as "E" medium to grow the molds. This medium was prepared by adding 1 per cent by volume of corn steep liquor (55 per cent solids) to Czapek's solution, adjusting the pH to 7.0, adding 2.0 per cent agar, distributing portions measuring exactly 20 ml. in tubes and sterilizing. The contents of each tube were poured into a sterile petri dish of 100-mm. diameter and of flat bottom, care being taken to use dishes that would provide a uniform depth of agar. A single colony was produced in the center of each plate as a result of inoculation with a small amount of a solidified suspension of the mold spores (prepared by suspending spores in melted agar at 45°C and allowing the agar to solidify). Control plates were prepared with two different cultures of *P. notatum* known to be good penicillin producers Each plate of the series was incubated for 6 days at 24°C

At the end of the incubation period, four or five plugs were cut and

[1] RAPER, K. B., D. F. ALEXANDER, and R D COGHILL, *Jour Bact.*, **48** (No 6) 639 (1944).
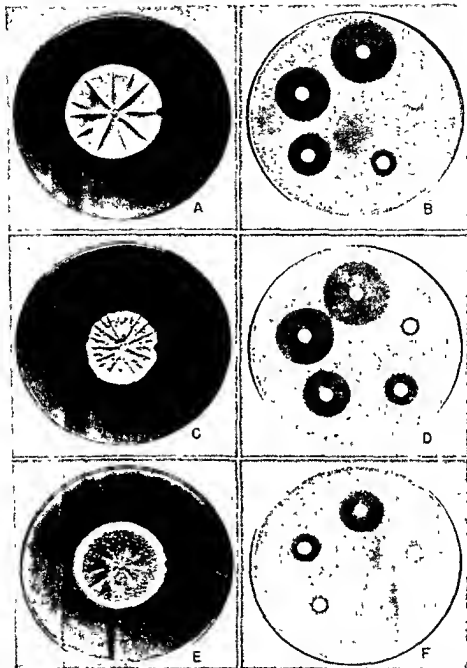
Fig 93 —Screening test   A, Control culture, *Penicillium notatum* NRRL 1249 B21, on "E" medium, incubation at 24 C   radial series of plugs cut at 6 days, × ⅔   B, Agar plug assay plate showing zones of inhibition of *Staphylococcus* developed after agar blocks removed from A have been incubated for 16 hours at 37 C, × ⅔   C, New isolate, good penicillin-producing strain, incubation and test as in A   D, assay plate for same.   F, New isolate, poor penicillin-producing strain, incubation and test as in A   F, Assay plate for same   [*Courtesy of K B Raper, D F Alexander, and R D Coghill, Jour Bact* 45 (No 6), 639 (1944)]

Wartime Developments.—In 1941, Florey and Heatley were invited to the United States by the Rockefeller Foundation. Conferences were held with Thom, principal mycologist of the U.S. Department of Agriculture, with personnel of the Committee on Medical Research of the Office of Scientific Research and Development, with the National Research Council, with the Northern Regional Research Laboratory, and with other groups. As a result of the visits and conferences, drug and chemical manufacturing concerns, for example, Merck and Company, E. R. Squibb and Sons, Charles Pfizer and Company, the Abbott Laboratories, and the Winthrop Chemical Company, became interested in the production of penicillin. Meetings of manufacturers were sponsored by Richards, Chairman of the Committee on Medical Research of the O.S.R.D., for the purpose of coordinating the effort, exchanging information, and speeding the development of the industry.

The rapid and extensive expansion of the penicillin industry was based on the cooperation, coordination, and vast amount of research of government agencies, of universities, of industrial laboratories, and of medical clinics in this country and England.

Properties of Penicillin.—Penicillin is active against certain Gram-positive bacteria in the presence of blood, pus, and body fluids.

It may be regarded as nontoxic, for very large doses may be administered over a relatively long period of time without injurious effects. In a small percentage of cases, individuals allergic to penicillin are found. Urticaria, hives, and itching are some of the responses observed in sensitive persons.

According to Welch and his associates,[1] there is partial inhibition of phagocytosis by penicillin.

It is soluble in water. In acetone, amyl acetate, cyclohexane, dioxane, ethyl acetate, ethyl alcohol, and ether, it is very soluble; in benzene, chloroform, and carbon tetrachloride, it is less soluble.

It is unstable under a number of conditions. For example, it is decomposed by strong acids and alkalies. It is inactivated by oxidizing agents. Contact with certain of the heavy metals, such as copper, silver, lead, and mercury   · · · · penicillin
Zinc salts may al: ·   · or-
zecanski.[2] The basic structure of penicillin is destroyed by · · · nol, especially methanol, by sulphydryl compounds, by heat, and by repeated freezing and thawing.[3]

Penicillin is destroyed by penicillinase, an enzyme secreted by a large

[1] WELCH, H., R. P. DAVIS, and C. W. PRICE, *Jour Immunol,* 51: 1 (1945).
[2] EISNER, H, and B. PORZECANSKI, *Science,* 103 (No. 2681). 629 (1946)
[3] RAKE, G., and A. P. RICHARDSON, *Ann. N. Y. Acad Sci,* 48 (Art. 2) 143 (1946).

number of bacteria, molds, and yeasts. The aerobic sporeforming bacteria and some of the actinomycetes are particularly destructive, Woodruff and Foster have reported.[1] Hence, it is necessary to use sterile media and aseptic methods during the production of penicillin and sterile water or other suitable diluents in the preparation of solutions of the solid material.

The crystalline salts of penicillin, especially when stored at low temperatures, are stable over a period of several months

Aqueous solutions of penicillin are unstable and must be stored under refrigeration

The pH of the aqueous solution containing penicillin has a very important bearing on the stability of the preparation. Penicillin is most stable in the pH range of 6 0 to 6.5 and reasonably stable over the pH range of 5.5 to 7.5. The half lives of penicillins F, G, K, and X are 11, 18 5, 7, and 11 min , respectively, at pH 2.0 and 24°C.

Phosphate has a stabilizing influence on crude penicillin at 37°C, according to Pratt,[2] who found that the rate of loss of potency varied inversely with the concentration of potassium dihydrogen phosphate present in the culture medium at the beginning. The stabilizing action of the phosphate was independent of the concentrations of the magnesium sulphate and sodium nitrate. Phenylacetic acid and *p*-hydroxybenzoic acid did not modify the stability of crude penicillin when present in the medium Pratt[3] demonstrated that low concentrations of a phosphate exerted a stabilizing influence on sterile-water solutions of penicillin, which was independent of the buffering effect.

Types of Penicillin.—Penicillins are compounds of the general formula $C_9H_{11}O_4SN$—R, in which R represents the radical or group that is different for each type The structural formulas of the most common types (F, G, X, and K) are given in Table 155

Most penicillins are mixtures, usually of types F, G, and K, but penicillin G is produced in largest quantities and considered to be the most practical Crystalline sodium penicillin G must contain at least 90 per cent of G. Type F penicillin was the predominant type produced during the early years of the World War II by surface-culture methods.

Source.—Penicillins F, G, X, and K are produced by strains of the *Penicillium notatum-chrysogenum* group of molds, flavicidin (flavicin), by *Aspergillus flavus*, and dihydro F penicillin (gigantic acid), by *A. giganteus*

Comparative Activities—The different types of penicillin vary in their

[1] Woodruff, H B , and J W Foster, *Jour Bact.*, 49 (No. 1) 7 (1945)

[2] Pratt, R , *Plant Physiol*, 22 (No. 3): 308 (1947).

[3] Pratt, R , *Jour Am Pharm. Assoc*, *Sci. Ed*, 36 (No. 3): 69 (1947).

activities towards different organisms, as will be evident by reference to Table 154 which shows the comparative activities of crystalline penicillins towards *Staphylococcus aureus* and *Bacillus subtilis*.

TABLE 154.—COMPARATIVE ACTIVITIES OF VARIOUS CRYSTALLINE PENICILLINS[1]

Penicillin type	Activity in units/mg. against		Ratio of activity vs. *B. subtilis*/ vs *Staph. aureus*
	Staph. aureus	*B. subtilis* R	
G (II)	1,667*	1,667*	1.0*
F (I)	1,465	970	0.65
Flavicidin	1,400	1,000	0.72
X (III)	850	1,450	1.4–2 0
K (IV)	2,300	760	0.33
Abbott No. 123	3,500		

[1] BENEDICT, R G., and A. F. LANGLYKKE, *Ann. Rev. Microbiol.*, 1: 193–236 (1947)
* By definition.

Relative Effectiveness.—The relative effectiveness *in vivo* of some of the penicillins, which depends on the kind of pathogen concerned, the rate of excretion, the extent of protein-binding in the blood, and other factors, has been studied by a number of scientists. Welch and his coworkers[1] found that penicillin X was more active than penicillin G against gonococci in man. Ory, Meads, and Finland[2] reported that two penicillin preparations, which contained 65 to 90 per cent of penicillin X, were two to eight times more active per unit towards strains of group A streptococci, gonococci, and meningococci. Libby and Holmberg[3] found that X (gravimetrically) was 1.2 to 2 times more active than G against the strains each of pneumococci and streptococci *in vitro*. Eagle,[4] on the basis of *in vitro* experiments, observed that the order of increasing activity for *Staphylococcus aureus* was X < F < G < K; for a streptococcus, F < G < K < X; and for *Spirochaete pallida*, X = F < K < G. Eagle and Musselman[5] obtained experimental data that indicated that penicillin K was inactivated in the body more rapidly and more extensively than penicillins F, G, and X and was thus of lower therapeutic value. On the basis of what they termed as preliminary and meager experimental evidence, Coghill and associates[6] concluded that penicillins

[1] WELCH, H., L. E PUTNAM, W. A. RANDALL, and R. P. HERWICK, *Jour. Am Med. Assoc.*, 126: 1024 (1944).
[2] ORY, E. M., M. MEADS, and M. FINLAND, *Jour. Am Med. Assoc.*, 129: 257 (1945).
[3] LIBBY, R. L., and N. L. HOLMBERG, *Science*, 102: 303 (1945)
[4] EAGLE, H , *Jour. Bact.*, 52: 81 (1946)
[5] EAGLE, H , and A. MUSSELMAN, *Science*, 103: 618 (1946).
[6] COGHILL, R. D., A. E. OSTERBERG, and G. R. HAZEL, *Science*, 103: 709 (1946)

TABLE 155 — CHEMICAL STRUCTURES OF SODIUM PENICILLINS

Type	British designation	Structure
Penicillin F (Δ²-pentenyl-penicillin)	I	$CH_2 \cdot CH_2 \cdot CH=CH \cdot CH_2$ ... $C(CH_3)_2$ ... $COONa$
Penicillin G (benzylpenicillin)	II	CH_2 ... $C(CH_3)_2$ ... $COONa$
Penicillin X (p-hydroxybenzyl-penicillin)	III	HO—⟨ ⟩—CH_2 ... $C(CH_3)_2$ ... $COONa$
Penicillin K (n-heptylpenicillin)	IV	$CH_3(CH_2)_6$ ... $C(CH_3)_2$ ... $COONa$
Dihydro F	Gigantic acid	$CH_3 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2$ ... $C(CH_3)_2$ ... $COONa$
Flavicidin Flavicin	Γ type	$CH_3 \cdot CH=CH \cdot CH_2 \cdot CH_2$ ... $C(CH_3)_2$ ... $COONa$

G and X were sufficiently stable, that the use of penicillin X for clearing certain types of susceptible infections was indicated; and that penicillin K was so unstable in human beings that there was serious question concerning its value for therapeutic purposes.

Production of Penicillin.—There have been a relatively large number of publications on penicillin production. Among them are the reports of Clutterbuck, Lovell, and Raistrick (1932); Challinor (1942); Challinor and MacNaughton, and Foster, Woodruff, and McDaniel (1943); Callahan; Coghill; Cook, Tulloch, Brown, and Brodie; Elder; Merck and Company; Pearl and Appling, Raper, Alexander, and Coghill, Sninivasa; and Waksman and Reilly (1944); Koffler, Knight, Perlman, and Burris; Koffler, Knight, Emerson, and Burris; Raper and Alexander; Raper and Coghill; and White, Krampitz, and Werkman (1945); Bowden and Peterson; Foster, Woodruff, and McDaniel; Foster, Woodruff, Perlman, McDaniel, Wilker, and Hendlin; Moyer and Coghill; Raper and Fennell; Stefaniak, Gailey, Brown, and Johnson, and Stefaniak, Gailey, Jarvis, and Johnson (1946); Moyer and Coghill, and Taylor (1947).

Fig. 94—Surface-culture production of penicillin by *Penicillium notatum* NRRL 1249 B21. (Photo by Cecil G. Dunn.)

Penicillin may be produced by both surface-culture and submerged-culture methods. In the surface-culture methods, the mold is grown on the surface of shallow layers of the fermentation or production medium or of bran on a laboratory, pilot-plant, or commercial scale. In the submerged-culture methods, the mold is grown submerged in the fermentation medium in shake flasks, rotary drums, or deep tanks. The shake flasks are usually used for laboratory studies, while the rotary drums and deep tanks are used for pilot-plant or commercial production. Agitation and aeration of the medium are essential in the submerged-culture processes.

Surface-culture Methods.—In the surface-culture methods, the mold grows at the surface of a shallow layer of medium as a mycelial mat. The oxygen required is taken from the air by the mold. No attempt is made to aerate or agitate the medium. Metabolic products formed move away from the surface by diffusion and fresh nutrients (while they last) are brought to the mold by the same process.

LABORATORY METHODS.—These methods have been used principally for the purpose of studying new strains of molds, the effects of various constituents of the medium on production, and the relationship of environmental factors on yields A sterile medium of suitable composition is inoculated with the spores, or germinated spores, of a selected strain of mold and incubated at a temperature optimum for the growth of the mold and the accumulation of penicillin.

A discussion of the molds used for the production of penicillin is presented in a later section. It will suffice to state here that a strain of mold belonging to the group known as *P. notatum-chrysogenum* is employed

Various media have been devised for the production of penicillin. One of fundamental importance is the following which was developed by Moyer and Coghill[1]

Corn-steep liquor	100 g	$MgSO_4 \cdot 7H_2O$	0 250 g
Lactose monohydrate	44 0 g	$ZnSO_4 \cdot 7H_2O$	0 044 g
Glucose monohydrate	2 75 g	$MnSO_4 \cdot 4H_2O$	0 001 g
$NaNO_3$	3 0 g	Water to make	1,000 ml
KH_2PO_4	0 500 g	Initial pH	4 6

Media, such as the foregoing, are dispensed into 200-ml Erlenmeyer flasks in 50-ml amounts, or into 300-ml. Erlenmeyer flasks in 75-ml. quantities, and sterilized at 15 lb steam pressure for 20 min.

The contents of the flasks are cooled to 28 to 24°C and inoculated with the mold spores The flasks are then incubated at a temperature favorable for the accumulation of penicillin, which is about 24°C. However, they may occasionally be incubated initially for less than 36 hr. at a temperature of 28°C. to encourage the rapid germination of the mold spores Highest yields are usually recovered after incubation of the flasks for 5 to 7 days

COMMERCIAL METHODS.—Large-scale production of penicillin was first carried out by surface-culture methods, which were essentially extensions of the methods employed in the laboratory. One of the most important

[1] MOYER, A J., and R D COGHILL, *Jour Bact*, 51 (No 1) 57 (1946)

strains of molds used in the early years of World War II for surface-culture production was *P. notatum* NRRL 1249.B21, a substrain derived from the Fleming strain and developed at the Northern Regional Research Laboratory.

The containers used for the production of penicillin included milk bottles, Fernbach flasks, conical flasks, special flasks of the type manufactured by the Glaxo Laboratories,[1] and other miscellaneous types. Milk bottles were popular since there were facilities for their fabrication in large numbers available and also machines for washing them.

The concerns that produced penicillin by the surface-culture method were often referred to as bottle plants or factories. At least one of these plants handled 750,000 bottles routinely.

Sufficient medium was introduced into each container to provide an optimum depth for penicillin production. The optimum depth of medium was usually about 2 cm., although it varied from 1 to 4 cm. Ordinarily the yield per flask was more important than the yield per milliliter under optimum conditions for maximum yields that necessitated the use of very shallow layers of medium.

The flasks or bottles with their contents were transported in wire or metal baskets by dollies or trollies to autoclaves, where they were sterilized. After the contents were cooled to 24°C., they were inoculated with dry mold spores, or aqueous suspensions of the spores, by means of pipettes or spray guns.

The inoculated flasks were incubated at 24°C. and maintained at this temperature until the penicillin was recovered, which was usually 5 to 8 days in the United States and 7 to 11 days in England.

It will be obvious that there is a very large amount of hand labor required for the operation of a bottle plant, since each bottle must be handled as a separate unit. Likewise a large amount of incubator space is required.

In the United States conversion to the submerged-culture method was started late in 1943 and now is the commercial process employed.

Submerged-culture Methods.—In the submerged-culture method, the mold grows throughout the medium in the form of pellets. Aeration and agitation of the medium are essential.

LABORATORY METHODS.—On a laboratory scale, shake-culture procedures are generally employed of which the following example is typical.

Moyer and Coghill[2] reported on the use of *Penicillium notatum* Westling (NRRL 832), parent of one of the substrains used in industry for the submerged-culture production of · ·   in prior to the su

[1] SMITH, E. L., *Jour. Soc. Chem. Ind.*, **65** 314 (1946).
[2] MOYER and COGHILL, *op. cit.*, p. 79.

1944, for the formation of penicillin on a laboratory scale. The following medium was devised and developed by them:

Corn-steep liquor[1] ..	40 0 ml	KH$_2$PO$_4$	0 50 g
Lactose monohydrate	27 5 g	ZnSO$_4$ 7H$_2$O	0 044 g
Glucose monohydrate	3 0 g	MnSO$_4$ 4H$_2$O	0 020 g
NaNO$_3$	3 0 g	Distilled water to make	1 liter
MgSO$_4$ 7H$_2$O	0 25 g		

[1] Commercial product containing 50 to 55 per cent solids (30° Bé)

The medium was dispensed into 300-ml Erlenmeyer flasks at the rate of 125 ml. per flask. The flasks plus their contents were sterilized in the usual manner, cooled, and inoculated with a suspension of mold spores, or with the dry spores or with germinated spores in the form of pellets, after each flask had received 1 to 1.5 g. of sterile calcium carbonate. The flasks were placed on the platform of a Rass-Kershaw shaking machine (other types are satisfactory) and caused to revolve at 200 revolutions or cycles per minute, according to Mayer and Coghill. The temperature was maintained at 24°C. The fermentations were generally complete in 6 to 7 days.

Mayer and Coghill found that the fermentation time could be shortened by using pregerminated spore inoculums These were prepared as follows: A medium containing the same salts and in the same concentrations as indicated above was used In addition to the nutrient salts, 55 ml. of corn steep liquor and 30 0 g of lactose per liter were employed. This medium was distributed in 300-ml Erlenmeyer flasks at the rate of 125 ml per flask, sterilized, and cooled One gram of sterile calcium carbonate was added to each flask, which was inoculated with mold spores The flasks with their contents were shaken on the Ross-Kershaw shaker for 2 to 3 days and then 5 to 7 5 ml of the medium containing the pellets were used to inoculate the production medium, according to Moyer and Coghill.

It was found that they could increase the yields of penicillin from the shake cultures by adding fresh nutrients to the production flasks, starting on the fourth day after inoculation and continuing daily thereafter for a few days One feed consisted of 5 ml containing 0 9 g of glucose and 0 2 ml of corn-steep liquor A maximum yield was obtained on the ninth day under such conditions in one experiment

PILOT-PLANT METHOD—The design and operation of pilot-plant equipment for the production of penicillin by a submerged-culture method has been described by Stefaniak and his associates,[1] at the University of

[1] STEFANIAK, J J., F B GAILEY, C S BROWN, and M J JOHNSON, *Ind Eng Chem.*, **38**: 666 (1946)

1 Steam-jacketed air sterilizer for seed tank
2 Seed tank
3 Measuring tank for inoculum
4 Inoculum line
5 Inoculum pressure gage
6 Air vent throttling valve
7 Exhaust air sampling cock,   7A. Safety valve
14 A, B, C, Masterilo valves

Fig. 95 — Flow diagram of penicillin pilot plant

8 Sampling valve
9 Drain valve
10 Steam-jacketed air sterilizer for fermenters
11 Fermenter
12 Vessel containing antifoam agent
13 Antifoam agent standpipe

15 Gate valves
16 Condensate valve
17 Drain valve for antifoam agent
18 Holding chamber for antifoam agent
19 Reducer
20 Check valve,   20A, check valve
21 Air flow regulating valve

381,600 (1946).]

[Courtesy of J. J. Stefaniak, P. B. Gailey, C. S Brown, and M. L. Johnson, Ind. Eng. Chem.

Wisconsin. A flow diagram of the apparatus is shown in Fig. 95. The fermentation tanks are of 100-gal. capacity.

The inoculum for the fermenter was prepared as follows by Stefaniak and his coworkers: Bottle-spore plates were inoculated from soil-stock cultures of the mold strain being used and incubated at 23°C. for 4 days. An aqueous suspension was made of the spores from one plate and used to inoculate two shake flasks, each of which contained 100 ml. of medium 1 (refer to Table 156). The shake flasks were incubated for 24 hr. at 23°C. and then their contents were used to inoculate the ingredients of the seed inoculation tank, which contained medium 2 (refer to Table 156). The contents of the seed tank were aerated with 100 liters of air per minute and maintained at 23°C. for 36 hr. A portion of this medium was blown to a measuring tank and 20 liters were used to inoculate the fermenter which contained 200 liters of medium 3 (refer to Table 156).

Fig. 95 —Fermentor assembly.*
[Courtesy of J. J. Stefaniak,
F. B. Gailey, C. S. Brown, and
M. J. Johnson, Ind. Eng. Chem.,
38: 666 (1946).]

The use of an antifoam agent and aeration are essential in submerged fermentation of this type. At the time when the fermenter was inoculated, 300 ml. of sterile antifoam agent (3 per cent of octadecanol in lard oil) were added by manual operation. The tank was aerated at the rate of 60 liters per min. during the first 6 hr., then at the rate of 200 liters per min. The automatic antifoam system was also placed into operation.

The effect of aeration and agitation on the production of penicillin by four different cultures is shown in Table 157. An aeration rate of 1 volume of air per minute for each volume of medium was found to be optimum by Stefaniak and his associates.[1]

COMMERCIAL METHODS.—Production details vary from plant to plant, but they are alike in the fundamentals. Special selected strains of molds belonging to the *Penicillium notatum-chrysogenum* group are used in all cases. One such mold is *P. chrysogenum* Wisconsin Q176. Deep tanks or vats, equipped with stirring, aerating, and other devices and with means for maintaining the temperature at the desired level, are employed

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* 1 Handhole, 2 foam-detecting electrode, 3 baffle plate, 4 agitator, 5 sparger, 6 cold water inlets
  [1] *Ibid.*

STEAM
STEAM
AIR
STEAM
COLD WATER
STEAM
STEAM
AIR

1. Steam-jacketed air sterilizer for seed tank
2. Seed tank
3. Measuring tank for inoculum
4. Inoculum
5. Inoculum pressure gage
6. Air vent throttling valve
7. Exhaust air sampling cock 7A. Safety valve
14. A, B, C. Magnetic valves

8. Sampling valve
9. Drain valves
10. Steam-jacketed air sterilizer for fermentors
11. Fermentor
12. Vessel containing antifoam agent
13. Antifoam agent standpipe
15, 16. Gate valves
17. Condensate valve
18. Drain valve for antifoam agent
19. Holding chamber for antifoam agent
20. Rotameter   20A. Check valve
21. Air flow regulating valve

Fig. 95.—Flow diagram of penicillin pilot plant.
(Courtesy of J. J. Stefaniak, P. B Gaden, C. S. Brown, and M. L. Johnson, Ind. Eng. Chem.
38: 666 (1946).)

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Fig 96—Fermentor assembly.*
[Courtesy of J J. Stefaniak, F B Gailey, C. S. Brown, and M J Johnson, Ind Eng Chem, 38: 666 (1946)]

The use of an antifoam agent and aeration are essential in submerged fermentation of this type. At the time when the fermenter was inoculated, 300 ml of sterile antifoam agent (3 per cent of octadecanol in lard oil) were added by manual operation. The tank was aerated at the rate of 60 liters per min during the first 6 hr, then at the rate of 200 liters per min. The automatic antifoam system was also placed into operation

The effect of aeration and agitation on the production of penicillin by four different cultures is shown in Table 157. An aeration rate of 1 volume of air per minute for each volume of medium was found to be optimum by Stefaniak and his associates [1]

COMMERCIAL METHODS—Production details vary from plant to plant, but they are alike in the fundamentals Special selected strains of molds belonging to the *Penicillium notatum-chrysogenum* group are used in all cases One such mold is *P chrysogenum* Wisconsin Q176 Deep tanks or vats, equipped with stirring, aerating, and other devices and with means for maintaining the temperature at the desired level, are employed

* 1 Handhole, 2 foam-detecting electrode, 3 baffle plate, 4. agitator, 5 sparger, 6 cold water inlets

[1] *Ibid*

TABLE 156.—MEDIA USED IN FERMENTATIONS[1,2]

Constituents	Medium 1 (shake flasks)	Medium 2 (inoculation tank)	Medium 3 (fermenter)	Medium 4 (fermenter)
Dextrin	60	.	.	.
Glucose	.	40	.	.
Lactose	.	......	30	20
Corn-steep solids	20	20	40	20
Sodium nitrate	.	3	..	3
Potassium phosphate[3]	.	0.5	..	0.5
Magnesium sulphate	..	0 125	..	0 125
Calcium carbonate	.	5	10	1.8

[1] STEFANIAK, J J., F B GAILEY, C S BROWN, and M J JOHNSON, *Ind. Eng. Chem.*, 38: 666 (1946).
[2] In grams per liter
[3] Monobasic

TABLE 157.—EFFECT OF AERATION AND AGITATION ON PRODUCTION OF PENICILLIN[1]

Culture	Medium	Type of stirrer	Stirring rate, r p m	Rate of aeration, l /min.	Maximum yield, units/ml	Time to maximum
P. notatum 832	4*	Sweep arm	30	50	29	11 days
P. notatum 832	4*		105	60	35	10 days
P. notatum 832	4*		105	65	40	8 days
P. notatum 832	1*	Marine propeller, 10 in diam (downward thrust)	350	60	35	7 days
P. notatum 832	4*		350	120	46	6 days
					49	3 days
P. notatum 832	1†	Flat blade propeller, 16 in diam (upward thrust)	250	170	62	58 hr
P. notatum 832	1†		270	275	99‡	71 hr
P. notatum 832	3†		270	200	245	64 hr
P. chrysogenum 1951 B25	3†		270	200	470	72 hr
P. chrysogenum X-1612	3†		270	200	737	66 hr
P. chrysogenum Q176	3†					

[1] STEFANIAK, J J., F B GAILEY, C S BROWN, and M J JOHNSON, *Ind. Eng. Chem.*, 38: 666 (1946).
* Five per cent inoculum used.
† Ten per cent inoculum used.
‡ Ninety-three units per milliliter at 59 hr.
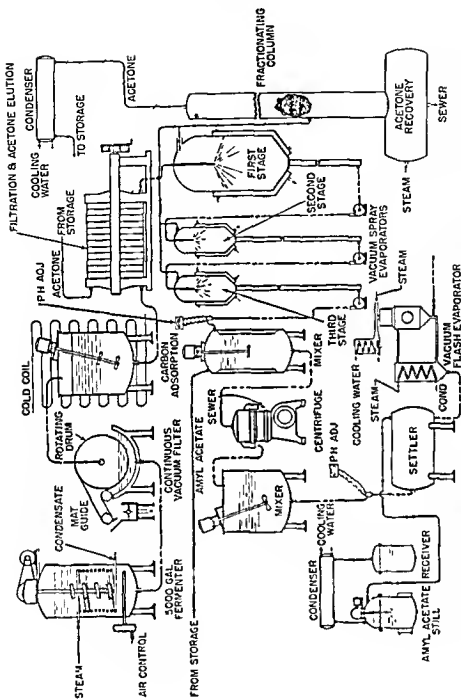
Fig. 97.—Penicillin-process flow diagram [*Courtesy of T. H. M. Taylor, Chem. Eng. Prog., 43 (No. 4): 155 (1947).*]

for the commercial production of penicillin.   Such tanks hold several thousand gallons of medium.   The medium used for the production of penicillin commonly contains corn-steep liquor, lactose, glucose, nutrient salts, phenylacetic acid derivatives (refer to page 743), and generally calcium carbonate, which aids in controlling the pH.   Modifications of the media are found in different plants and are made whenever it may be shown that improvement in the process results therefrom.

Figure 97 shows the flow diagram for industrial production in one plant.

Penicillin X.—The production of penicillin X in submerged culture has been described by Raper and Fennell.[1] The mold used by them was *P. chrysogenum* NRRL 1984.N22, an ultraviolet mutant of NRRL 1984.A (a substrain of Minnesota R-13).   The medium contained 2.5 per cent of lactose, 6 per cent (by volume) of corn-steep liquor, 0.5 per cent of calcium carbonate, and tap water to 600 liters.   After sterilization and cooling, it was inoculated with 12 liters of the germinated spores of the mold, aerated at the rate of 600 liters per min., and agitated at 250 r.p.m.   The temperature was kept at 24 to 25°C.   The fermentation was completed in 90 hr.   Approximately 50 per cent of the penicillin activity of the medium was due to penicillin X.

For production by the shake-culture procedure, Raper and Fennell[1] used the following medium and method:

Lactose	20 0 g	KH₂PO₄	0 5 g
Corn-steep liquor (conc)	40 0 ml.	MgSO₄.7H₂O	0 25 g
Cerelose (commercial glucose)	1 2 g	Distilled water to.	1 liter
Na₂NO₃	3 0 g		

The foregoing medium was distributed into 1-liter Erlenmeyer flasks at the rate of 200 ml. per flask and sterilized.   Sterile calcium carbonate (1.6 g.) and 2 drops of sterile lard oil were added to each flask.   The contents of each container were then inoculated with a spore suspension consisting of approximately 20 to 40 million spores.   The flasks were agitated on a rocker-type shaker, which produced one hundred 3-in. strokes per minute, during incubation at 24 to 25°C.

The production of penicillin X by a "submerged" surface-culture method has been described by Stice and Pratt.[3] *P. notatum* 1249.B4, a variant of *P. notatum* NRRL 1249.B21, was employed by them.

Molds Used.—Considerable research concerning the selection and development of strains of molds particularly suited for penicillin produc-

[1] RAPER, K. B , and D. I. FENNELL, *Jour. Bact.*, **51** (No. 6): 761 (1946).

[2] *Ibid.*

[3] STICE, E , and R. PRATT, *Science*, **103** (No 2678): 535 (1946).

TABLE 158.—INFORMATION CONCERNING SOME MOLDS USED FOR PENICILLIN PRODUCTION[1]

Designation of mold	History	Use
Penicillium notatum, NRRL 1249 B21	Substrain derived from Fleming strain and developed at NRRL. More widely used than any other strain for surface-culture production in U S and abroad	Surface-culture production in industry
P notatum, NRRL 832 A2	Substrain of NRRL 832 max yields of 100 units/ml	Submerged-culture production in industry prior to summer of 1944
P chrysogenum, NRRL 1951 B25	Substrain of NRRL 1951 Yields in excess of 200 units/ml Produced higher yields of penicilin in submerged culture than NRRL 832	Submerged-culture production in industry during latter half of 1944 and early 1945
P chrysogenum, Stanford 25099	Substrain of NRRL 1951 B25 resulting from ultraviolet radiation (Prof G W Beadle)	Superior submerged-culture production
P chrysogenum, Stanford 35217	Substrain of NRRL 1951 B25 resulting from ultraviolet radiation. (Prof G W Beadle)	Limited production by submerged culture in industry
P chrysogenum, X-1612	Mutation of NRRL 1951 B25 induced by X rays by Dr M Demerec and associates at Carnegie Institute of Washington Yields in excess of 500 units/ml	Submerged-culture production (superior strain)
P chrysogenum, NRRL 1981	Isolated from soil in Minnesota Minn R-13 (Prof C M Christensen)	The parent of commercially important strains used for submerged-culture production
P chrysogenum, Minn 9-88251	Substrain of Minn R-13	Submerged-culture production (high yields)
P chrysogenum, NRRL 1981 A	Substrain of NRRL 1981 (Minn R-13) Greater yields than parent Yields to 260 units/ml Best producer of penicilin X	Submerged-culture production in industry
P chrysogenum, NRRL 1981 X22	Substrain of NRRL 1981 A, resulting from treatment of conidia with ultraviolet radiation About one-half of penicillin units are penicillin X	Submerged-culture production of Penicillin X

[1] See footnote at end of table

TABLE 158.—INFORMATION CONCERNING SOME MOLDS USED FOR PENICILLIN PRODUCTION[1].—(Continued)

Designation of mold	History	Use
P. chrysogenum, Q176 (Wisconsin)	Substrain of *P. chrysogenum* X-1612 resulting from treatment of conidia with ultraviolet light (Prof. M. P. Bakus and Dr J. F. Stauffer, Univ. of Wisconsin). Yields in excess of 761 units/ml	Submerged-culture production

[1] Based principally on information contained in articles by K. B. Raper and D. I. Fennell *Jour Bact*, 52 (No. 6): 761 (1946) and K B Raper and D. F. Alexander [*Jour. Elisha Mitchell Scient Soc.* 61 (Nos 1 & 2): 74 (1945)]

tion has been carried out, especially by Raper, Alexander, and Coghill;[1] Raper and Alexander;[2] Raper and Fennell;[3] and Backus, Stauffer, and Johnson.[4]

Raper, Alexander, and Coghill[1] studied 241 different cultures of molds, which were members of the *P. notatum-chrysogenum* group, for penicillin production. They concluded that penicillin production was characteristic of the whole *P. notatum-chrysogenum* group, that good production was usually limited to strains of *P. notatum* Westling and *P. chrysogenum* Thom, and that strains varied considerably in their capacities to produce penicillin.

Raper and Alexander,[2] over a 2-year period, investigated natural variation and penicillin production in four members of the *P. notatum-chrysogenum* group being used commercially for the production of penicillin. These included the original Fleming isolate, *P. notatum* Westling, and strains developed from it, for example NRRL 1249.B21, a strain widely employed for penicillin production by the surface-culture method; *P. notatum* NRRL 832, used for penicillin production by the submerged culture method; *P. notatum* NRRL 1950, and strains derived from it that produced high yields of penicillin in surface culture; and *P. chrysogenum* Thom NRRL 1951.B25, used for penicillin production in both surface- and submerged-culture methods. The various strains are described in detail in their significant paper.

In Table 158 is summarized information concerning strains of molds used for penicillin production.

[1] RAPER, ALEXANDER, and COGHILL, *loc. cit.*

[2] RAPER, K. B., and D. F. ALEXANDER, *Jour. Elisha Mitchell Sci. Soc.*, 61: 74 (1945).

[3] RAPER, K. B., and D. I. FENNELL, *loc cit.*

[4] BACKUS, M. P., J. F. STAUFFER, and M. J. JOHNSON, *Jour. Am. Chem. Soc.*, 68: 152 (1946).

SPORULATION MEDIA —In the production of penicillin, it is necessary to grow rapidly large numbers of spores for inoculation or seeding purposes. A number of media have been developed for that purpose, of which a few will be described.

According to Moyer and Coghill,[1] good results may be obtained with the following medium:

SPORULATION MEDIUM

Glycerol	7 5 g
Cane molasses (edible quality as commonly sold at retail)	7 5 g
Corn-steep liquor	2 5 g
$MgSO_4 7H_2O$	0 050 g
KH_2PO_4	0 060 g
Peptone	5 00 g
NaCl	4 00 g
Fe-tartrate	0 005 g
$CuSO_4 5H_2O$	0 004 g
Agar	2 50 g
Distilled water to make	1 0 liter

The foregoing medium may be solidified by adding sufficient agar to bring the total amount to at least 15 to 25 g. per liter.

They[1] also described an alternate method for producing mold spores. In this method, fresh whole-wheat bread (free of mold inhibitors) is cut into 1-cm. cubes and steam-sterilized in shallow layers in Erlenmeyer flasks. The cooled bread is heavily inoculated with mold spores and incubated at 25 to 27°C. for 4 to 5 days. After the bread cultures have partially dried, they may be stored at 40°C. for as long as 2 weeks satisfactorily. The spore-covered bread may be crushed usually to a powdery mass after 6 to 7 days and used for inoculation purposes. As an alternative, it may be mixed with three to four volumes of a mixture of equal parts of sterilized whole-wheat flour and finely ground oat hulls

Wheat bran moistened with a 2 per cent solution of corn-steep liquor may also be used for producing spores, according to Moyer and Coghill.[2]

A sporulation medium of the following composition has been developed by Koffler and his associates.[3]

Agar	15 00 g	KH_2PO_4	0 10 g
Sugar-beet molasses	5 00 g	$MgSO_4 7H_2O$	0 05 g
Peptone	5 00 g	Distilled water to make	1 liter
NaCl	4 00 g		

[1] MOYER and COGHILL, *op cit*, p 57
[2] MOYER and COGHILL, *op cit*, p 79
[3] KOFFLER, H S, G KNIGHT, W C FRAZIER, and R H BURRIS, *Jour. Bact.*, 51 385 (1946)

Foster and his coworkers[1] used the following sporulation medium:

Sucrose or brown sugar	20 g	$MgSO_4 \cdot 7H_2O$	0 5 g
NaNO$_3$	6 g.	CaCl$_2$	25 g
KH$_2$PO$_4$	1 5 g.	Water, tap or distilled.	1 liter

NOTE. Aeration and agitation are supplied by shaking the flasks containing the inoculated medium on a rotary shaker

INOCULATION METHODS.—There are a number of methods used to inoculate or seed the various media employed in the manufacture of penicillin, a few of which will be mentioned here. In surface-culture methods, the surface of the medium is inoculated with dry spores, either alone or mixed with such materials as wheat flour or finely ground oat hulls, as has been described by Moyer and Coghill.[2] The spore material is applied by means of the inoculation loop, the spatula, atomizer or other method, the surface being covered as uniformly as possible.

The sporulation medium may be heavily inoculated with spores and then thoroughly mixed. It should, of course, be exposed in shallow layers.

In submerged-culture methods, the production medium may be inoculated with dry spores, by ungerminated spores in suspension, or by pellet inoculums. Suspensions of ungerminated spores may be prepared in sterile 0.1 per cent soap solutions,[3] in sterile water containing 100 p.p.m. (1 10,000) of sodium lauryl sulphonate,[4] in sterile aqueous solutions containing some other wetting agent, which must be nontoxic to the mold and penicillin, or by other means. The wetting agents aid in obtaining more uniform suspensions than can be secured with ordinary sterile water. The suspensions are introduced into the production medium by means of pipettes, spray guns, bazookas, or other means. The suspensions are uniformly mixed with the medium by agitation and aeration.

Pellet inoculums are those obtained by growing mycelium from mold spores under submerged conditions. The pellets are commonly used for inoculation purposes 2 or 3 days after the medium has been seeded with spores. Time is saved in the production stage by using pellet inoculations.

Raw Materials.—In the selection of the raw materials for the production of penicillin, it is essential to consider the nutritive requirements of

[1] FOSTER, J. W., L. L. McDANIEL, H B. WOODRUFF, and J L STOKES, *Jour Bact.,* 50: 365 (1945).

[2] MOYER and COGHILL, *op cit*, p. 57.

[3] *Ibid.*, p 79

[4] RAPER, ALEXANDER, and COGHILL, *loc cit*

the mold, the conditions for the optimum accumulation of penicillin, and the subsequent extraction and purification of the penicillin.

CARBON SOURCES.—Of the carbon sources investigated to date, none has proved to be superior to lactose. This conclusion was reached by Moyer and Coghill,[1] Stone and Farrell,[2] and others. In 3 per cent concentrations lactose, corn starch, and corn dextrin were of approximately similar value for producing penicillin in media containing corn-steep liquor and the standard salts, according to Moyer and Coghill,[1] but in 6 per cent concentration lactose produced the highest yields. Glucose, sucrose, glycerol, and sorbitol were inferior as carbon sources. Moyer and Coghill attributed the superiority of the lactose (and starch) to the fact that the pH range was more favorable for fungus growth and penicillin accumulation over a longer time than was the case with glucose and the other carbon sources

NITROGEN SOURCES.—Sodium nitrate has been widely used in fermentation media, both for surface-culture and submerged-culture production of penicillin. The usual concentration employed is 3 g. per liter. Moyer and Coghill[3] found this salt to be better than ammonium salts or urea for the submerged production of penicillin

Corn-steep liquor supplies organic nitrogen to the medium.

Stone and Farrell[2] reported that some amino or ammonia nitrogen was essential in synthetic media for penicillin production, but that too much of either reduced the yields

MINERALS SOURCES.—The mineral requirements for mold growth and penicillin production have been investigated by Pratt,[4] Moyer and Coghill,[1,5] Stone and Farrell,[7] Foster and Associates,[8] and others.

Certain elements (phosphorous, potassium, magnesium, sulphur, zinc, and copper) appear to be necessary or beneficial for the production of penicillin, but some of them are to be found in the corn-steep liquor. Potassium and phosphorous are customarily supplied as potassium dihydrogen phosphate; magnesium and sulphur, as magnesium sulphate ($MgSO_4, 7H_2O$) Iron and copper, when added to the medium, are usually supplied as the sulphates. According to Moyer and Coghill,[6] the

[1] MOYER and COGHILL, *op cit*, p 57
[2] STONE, R W, and M A FARRELL, *Science*, 104 (No 2706) 445 (1946).
[3] MOYER and COGHILL, *op cit*, p 79
[4] PRATT, R, *Am. Jour Bot*, 32 (No 8) 528 (1945)
[5] MOYER and COGHILL, *op cit*, p 57
[6] *Ibid*, p 79
[7] STONE and FARRELL, *loc cit*
[8] FOSTER, J W, H B WOODRUFF, and L E McDANIEL, *Jour Bact*, 51 (No 4) 465 1946)
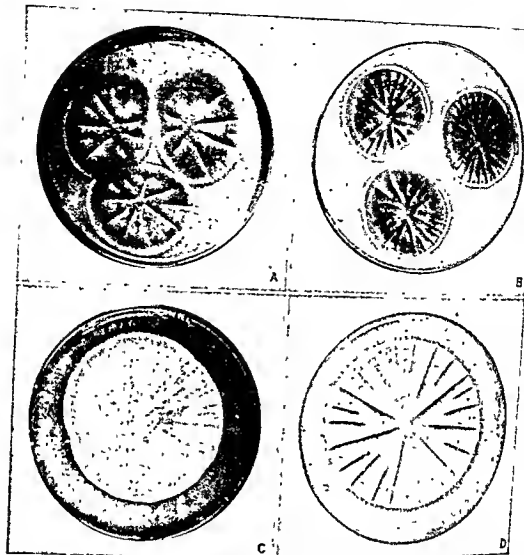
Fig. 98.—Penicillin-producing strains of *Penicillium notatum*. *A*, NRRL 824, the Fleming strain; *B*, NRRL 1249, the Squibb strain; *C*, NRRL 1249 B21, the strain most widely used for surface-culture prod. ... ... *D*, NRRL 832, ... rain used for submerged *A* grown on Czapek's
[*Courtesy of K B Raper*]
r 74 (1945)]

addition of zinc or manganese ions to a medium containing corn-steep liquor is optional. When used, they are supplied as the sulphates.

Corn-steep liquor appears to contain adequate quantities of KH_2PO_4 and $MgSO_4 \cdot 7H_2O$, according to Moyer and Coghill,[1] and Foster and associates.[2] However, small amounts of these salts may be added to the production medium.

[1] MOYER and COGHILL, *op. cit.* p 57.
[2] FOSTER, J. W., H. B. WOODRUFF, and L. E. McDANIEL, *Jour. Bact.*, 51 (No. 4): 465 (1946).

Pratt[1] studied the influence of various proportions of KH_2PO_4, $MgSO_4 \cdot 7H_2O$, and $NaNO_3$ on penicillin production by *P. notatum* in media containing lactose, corn-steep liquor, zinc sulphate, and phenyl-acetic acid. He found that the absolute concentrations of these salts in the best solution were KH_2PO_4, 0.019 M; $MgSO_4 \cdot 7H_2O$, 0.002 M; and $NaNO_3$, 0.019 M (a total molar concentration of 0.040).

CORN-STEEP LIQUOR.—The use of corn-steep liquor in the fermentation medium greatly increases the yields of penicillin. This fact was demonstrated in respect to the surface-culture and submerged-culture production of penicillin by Moyer and Coghill.[2,3]

Corn-steep liquor, or steep water, is a nutrient extract obtained during the manufacture of starch and other corn products. The role of this material in the production of penicillin has been studied by Bowden and Peterson[4] who reported that corn-steep liquor samples from different producers showed the following analyses:

	Per cent		Per cent
Solids	40–60	Amino nitrogen[1]	2.6–3.3
Lactic acid[1]	12–27	Reducing sugars, as glucose[1]	1.5–11
Total nitrogen[1]	7.4–7.8	Ash[1]	18–20

[1] On a dry basis

There is considerable variation in penicillin yields from samples from different manufacturers and in lots from the same manufacturer, according to Bowden and Peterson.[4]

The optimum concentration of corn-steep solids in shake cultures was found by Bowden and Peterson to be 2 per cent for both *P. notatum* NRRL 832 and *P. chrysogenum* X-1612. However, a concentration of 4 per cent was observed to be optimum when larger volumes of medium were used with aeration and agitation.

Cuban high-test molasses, steffanized molasses A and B, Bacto peptone, Difco yeast extract, solubilized liver, asparagus-butt juice, grass juice powder, rice steep, cottonseed meal extract, ground liver, and liver infusion were found to be of little value as corn-steep liquor substitutes. Although meat-scraps meal was somewhat superior to most of the foregoing substances, it was inferior to corn-steep liquor, according to Bowden and Peterson

[1] Pratt, R., *Am. Jour. Bot.*, **32** (No. 8, 528 1945).
[2] Moyer and Coghill, *op. cit.*, p. 57.
[3] *Ibid.*, p. 79.
[4] Bowden, J. P., and W. H. Peterson, *Arch. Biochem.*, **9** (No. 3) 387 (1946).

Foster and associates[1] reported that the stimulatory effects of corn-steep liquor were due in part to arginine, histidine, and glutamic acid and possibly to phenylacetic acid derivatives.

Koffler and collaborators[2] investigated the effect of certain mineral elements on penicillin production by *P. chrysogenum* X-1612 in shake flasks. They found that 0.5 g. of corn-steep ash (equivalent to 3 per cent of corn-steep solids) was required for the maximum production of penicillin; that the ingredients of the insoluble fraction of the ash were largely responsible for the stimulatory effect on penicillin production but that both the soluble and insoluble fractions of the ash were essential for maximum yields of penicillin; that the physiological function of corn-steep ash was due to the iron and soluble phosphates; that iron was stimulatory to a high degree by itself; and that phosphates demonstrated hardly any effect in the absence of iron. Chromium and iron were found to be able to increase the yields of penicillin nearly as much as iron and phosphates.

TABLE 159.—COMPARISON OF CORN-STEEP LIQUOR AND COTTONSEED MEAL WITH TWO DIFFERENT STRAINS OF *P. chrysogenum*[1,2]

Medium	Demeric strain X1612				Wisconsin strain Q176			
	3 days	4 days	5 days	6 days	3 days	4 days	5 days	6 days
2 per cent corn-steep solids, 3 per cent lactose, 1 per cent CaCO₃	140	180	200	120	375	550	650	600
4 per cent cottonseed meal, 3 per cent lactose, 1 per cent CaCO₃	190	250	300	175	520	800	950	1,000
Corn-steep liquor medium plus 0.05 per cent phenylacetyl derivative	160	250	350	300	1,050	1,300	1,250	900
Cottonseed meal medium plus 0 05 per cent phenylacetyl derivative	160	270	320	200	540	950	1,160	885

[1] FOSTER, J W , H B WOODRUFF, D PERLMAN, L E McDANIEL, B L WILKER, and D. HENDLIN Jour Bact. 51 : 695 (1946)

[2] Data represent Oxford units per milliliter and are averages of triplicate flasks, submerged cultures on rotary shakers, 80 ml of medium per 250-ml Erlenmeyer flask, temperature—23 to 25°C

COTTONSEED MEAL.—Cottonseed meal was found by Foster and his associates[1] to be equal to or superior to corn steep liquor for penicillin production by *P. chrysogenum* Demeric X-1612 and *P. chrysogenum*

[1] FOSTER, J. W , H B. WOODRUFF, D. PERLMAN, L E. McDANIEL, B L. WALKER, and D HENDLIN, *Jour Bact.* 51 : 695 (1946)

[2] KOFFLER, H., S. G. KNIGHT, and W. C. FRAZIER, *Jour. Bact.*, 52 (No. 1): 115 (1947).

Wisconsin Q176. It stimulated production by *P. notatum* NRRL 832 but was inferior to corn-steep liquor for this strain.

Table 159 compares penicillin production by two different strains of *P. chrysogenum* when corn-steep liquor and cottonseed meal were used.

PHENYLACETIC ACID DERIVATIVES —The effect of phenylacetic acid derivatives on the formation of penicillin by various molds has been studied by Moyer and Coghill;[1] Higuchi, Jarvis, Peterson, and Johason;[2] Stone and Farrell,[3] and by others.

Phenylacetic acid and such derivatives as β-phenylethylamine and phenylacetamide when present in the fermentation medium in suitable concentration act as precursors to increase the amount of penicillin G produced These substances also increase the over-all quantity of penicillin formed.

Moyer and Coghill[1] showed that phenylacetic acid markedly increased the yields of penicillin by *P. notatum* NRRL 1249.B21 in surface culture, and by *P. notatum* NRRL 832 and *P. chrysogenum* NRRL 1951.B25 in submerged cultures Amounts ranging between 0.2 and 0 8 g. of phenylacetic acid per liter of culture medium appeared to be optimum for penicillin production

Since phenylacetic acid is toxic to the molds under certain conditions, for example, at low pH values, it is necessary to observe certain precautions in connection with its use Moyer and Coghill[1] found that the toxicity could be overcome by increasing the pH of the medium to 5 0 to 5 8 before inoculation or by adding the phenylacetic acid to the culture after the pH had risen above the critical value

Higuchi and his associates (1916) determined the effect of added phenylacetic acid derivatives on penicillin production by *P. chrysogenum* Q176 Information concerning their results and the fermentation media used by them is summarized in Table 160. Study of this table leads to the following conclusions. *P. chrysogenum* Q176 produced large proportions of penicillin K in the absence of phenylacetic acid derivatives (as high as 88 per cent in one run) The addition of phenylacetic acid derivatives greatly increased the proportions of penicillin G produced, particularly during the early hours of the fermentation. The over-all yields were also increased by the phenylacetic acid derivatives, especially by phenylacetic acid Corn-steep liquor, a source of these derivatives, likewise increased the proportion of penicillin G and the total amount of penicillin formed p-Hydroxyphenylacetic acid increased the quantities

[1] MOYER, A J, and R D COGHILL, *Jour Bact* 53 (No 3) 329 (1947)

[2] HIGUCHI, K, F G JARVIS, W H PETERSON, and M J JOHNSON, *Jour Am Chem Soc*, 68 (No 9) 1669 (1916)

[3] STONE and FARRELL, *loc cit*

TABLE 160.—EFFECT OF PHENYLACETIC ACID DERIVATIVES ON PENICILLIN PRODUCTION[1]

Run no.[2]	Compound added[4]	Age of fermentation, hr.	Total penicillin, units/ml	Percentage composition of penicillin, in yields		
				G	X[3]	K
1	None	42	94	13	..	87
		66	233	14	..	87
		75	264	12	..	88
	β-Phenylethylamine	42	117	91	..	9
		66	272	76	..	24
		90	435	66	..	34
	Phenylacetic acid[5]	42	169	88	..	12
		66	333	79	..	21
		75	387	74	..	26
	Phenylacetamide	42	110	91	..	9
		66	104	69	..	31
		90	384	57	..	43
2	None	60	161	44	3	53
		108	569	29	1	70
	β-Phenylethylamine	60	267	93	2	5
		108	726	78	5	17
	Phenylacetamide[5]	60	335	103	1	-4
		108	616	78	2	20
	Phenylacetic acid[5]	60	448	109	3	-12
		108	673	76	-1	25
	p-Hydroxyphenylacetic acid	60	195	57	18	25
		108	422	42	11	47
	p-Hydroxyphenylacetic acid[5]	60	209	39	26	35
		108	462	34	10	56
3	None	49	638	30	..	65
4	Phenylacetic acid[6]	24	216	35	..	33
		60	1000	67	..	33
		72	1045	77	..	23

[1] Higuchi, K., F. G. Jarvis, W. H. Peterson, and M J Johnson, Jour. Am Chem Soc , 68 (No 8): 1669-1670 (1946).

[2] Runs 1 and 2 were carried out in 500-ml Erlenmeyer flasks containing 85 ml. (run 1) or 100 ml ... grams per liter ... magnesium chloride, ... following ... Runs ... 220 liters.

constitute ... 3 and 4
The medium used in run 3 contained in grams per liter: steep liquor solids, ... carbonate, 10, sodium sulphate, 1 0   The medium used in run 4 was the same as that in run 3 except that the steep liquor concentration was 20 g per liter.
[3] When the differential assay results were calculated as a mixture of G and K only, no figure is given for X.
[4] The compounds were added at a level of 0 5 g per liter, and were added before sterilization unless otherwise stated.
[5] The compound was added 24 hr. after inoculation
[6] The phenylacetic acid was added in six equal portions, at 0, 12, 24, 36, 48, and 60 hr.

of penicillin X produced, but, according to Higuchi and his associates, it appeared to be less readily available to the mold than phenylacetic acid.

Stone and Farrell (1946) demonstrated that the addition of 0.04 per cent of phenylacetamide or β-phenylethylamine increased substantially the total penicillin production by *P. chrysogenum* X-1612 in shake cultures.

CALCIUM CARBONATE —Calcium carbonate is customarily a constituent of the medium used for the submerged-culture production of penicillin. The concentration used is generally 1 per cent, according to Raper and Alexander,[1] Moyer and Coghill,[2] Stefaniak and associates,[3] Johnson,[4] and Foster and associates.[5]

SYNTHETIC MEDIA.—Studies have been made with the use of synthetic media for the production of penicillin. Such media, provided that they produce high yields, have the advantage of simplifying the extraction and purification of the penicillin. They also lend themselves to the study of the particular effects of an adjunct.

Synthetic media must contain adequate sources of carbon, nitrogen, phosphorous, potassium, magnesium, sulphur, iron, zinc, and copper, according to Stone and Farrell.[6] The latter found that lactose was the best source of carbon, that amino or ammonia nitrogen was essential, and that all satisfactory media contained some organic acid. Optimum results were obtained with acetic acid, but mixtures of acetic and formic acids or acetic and lactic acids were nearly as good.

The basal medium used by Stone and Farrell[6] for surface-culture studies contained the following ingredients

	Per cent		Per cent
Lactose	4	MgSO₄·7H₂O	0.025
Glacial acetic acid[a]	0.5	FeSO₄·7H₂O	0.02
NH₄NO₃	0.5	ZnSO₄·7H₂O	0.001
KH₂PO₄	0.1	CuSO₄·5H₂O	0.0005
NaNO₃	0.5		

[a] Or equivalent amount of potassium acetate

The pH of this medium was 6.1 before sterilization and finally above 5.6

[1] RAPER and ALEXANDER, *loc. cit.*
[2] MOYER, A. J., and R. D. COGHILL, *Jour. Bact.*, 51 (No. 1) 79 (1946)
[3] STEFANIAK, GAILEY, BROWN, and JOHNSON, *loc. cit.*
[4] JOHNSON, M. J., *Ann. N. Y. Acad. Sci.*, 48 (Art. 2) 57 (1946)
[5] FOSTER, WOODRUFF, PERLMAN, McDANIEL, WILKER, and HENDLIN, *loc. cit.*
[6] STONE and FARRELL, *loc. cit.*

Yields were increased by the addition of 0.4 per cent phenylacetic acid and were further augmented by the addition of 0.4 per cent phenylacetic acid and 0.1 per cent cysteine or cystine.

Conditions of Fermentations.—Under this heading may be considered conveniently such factors as temperature, pH, aeration, agitation, the use of antifoam agents, and the prevention of the contamination of the medium.

TEMPERATURE.—The optimum temperature for penicillin production by both surface-culture and submerged-culture methods is about 24°C; Moyer and Coghill[1] employed incubation temperatures of 24°C. ±1. Raper and Alexander,[2] 24°C.; and Stefaniak and associates,[3] a temperature of 23°C. for submerged-culture production by *P. notatum* 832, and *P. chrysogenum* strains NRRL 1951.B25, X-1612, and Q176.

Stefaniak and his coworkers[4] investigated the effect of temperature on yields of penicillin by *P. chrysogenum* X-1612 in submerged-culture production, using a medium containing 3 per cent of lactose, 4 per cent of corn steep liquor solids, and 1 per cent of calcium carbonate. They found that temperatures of 20 to 29°C. did not appreciably affect penicillin yields at 50 hr., but that a temperature of 32°C. produced definitely lower yields.

pH —The effect of pH on the production of penicillin has been studied by Moyer and Coghill,[5,6] Raper and Alexander,[7] Foster and associates,[8] Stone and Farrell,[9] Johnson,[10] Stefaniak and associates,[11] and others.

Control of the pH during the fermentation is essential, for too high or too low a pH may result in considerable losses of penicillin. According to Moyer and Coghill, the loss of penicillin may be substantial outside of the range of pH 5 to 7 5   In a normal fermentation, the pH rises from 4 or above to around 8.

The pH of the fermentation medium is influenced by the kind and

[1] MOYER, A J , and R D COGHILL, *Jour. Bact* , 51 (No 1) 57, 79 (1946).

[2] RAPER and ALEXANDER, *loc. cit.*

[3] STEFANIAK, GAILEY, BROWN, and JOHNSON, *loc. cit.,*

[4] STEFANIAK, J J , F B GAILEY, F G JARVIS, and M. J. JOHNSON, *Jour. Bact* 52 (No. 1). 119 (1946).

[5] MOYER, A. J., and R. D. COGHILL, *Jour Bact.*, 51 (No. 1): 57 (1946).

[6] *Ibid.*, p. 79.

[7] RAPER and ALEXANDER, *loc. cit.*

[8] FOSTER, WOODRUFF, and McDANIEL, *loc cit*

[9] STONE and FARRELL, *loc. cit*

[10] JOHNSON, *loc. cit.*

[11] STEFANIAK, GAILEY, BROWN, and JOHNSON, *loc. cit.*

amount of carbohydrate and the amount of corn-steep liquor present, by the presence of buffers, and other factors

Carbohydrates are generally fermented with the production of some organic acids. Some carbohydrates, such as glucose, are fermented rapidly and tend to lower the pH level; others, such as lactose, are fermented more slowly and consequently the pH changes more slowly. Large concentrations of fermentable sugars tend to retard the pH rise in the medium. In a submerged medium containing corn-steep liquor, lactose, and calcium carbonate, the mold utilizes the carbon from the nitrogen-containing portions of the corn-steep liquor in preference to the lactose at the beginning of the fermentation, according to Johnson.[1]

Fermentation of corn-steep liquor raises the pH of the medium, for the lactic acid is consumed

Likewise, utilization of the carbon from the amino acids liberates ammonia which acts to raise the pH level

Buffer agents tend to maintain the pH within a range favorable for penicillin production. Calcium carbonate is widely used for this purpose. When added to the medium, it raises the initial pH and tends to resist the raising of the pH to high final values. Calcium carbonate is not used in surface-culture production for it decreases the growth of the mold and the yield of penicillin

Moyer and Coghill found the optimum concentration of lactose in the medium used for the submerged production of penicillin to be between 2 and 3 per cent In a medium containing 2 per cent of lactose, the pH rose from 7 6 to 8 1 between the third and seventh days, and in a medium containing 3 per cent of lactose the pH rose from 7 6 to 7 8 between the third and seventh days

AERATION —Adequate aeration is essential for optimum yields of penicillin, for the molds are aerobic organisms and energy for synthetic processes, including the building of cells, is aided by the presence of available oxygen In shake-flask cultures, aeration is supplied by the rotary motion of the shaker, in the deep tank, it is supplied by forcing air through the medium, by means of agitation, and occasionally by the use of air under pressure

The requirements for a given tank will depend upon the diameter of the tank, the depth of the medium, the nature of the medium, the design of the aerating device (sparger), the design of the agitator, and other factors They must be determined for each installation Foster and associates[2] used an airflow of 150 cu ft per hr, an air pressure of 5 lb, and

[1] JOHNSON, *loc cit*
[2] FOSTER, WOODRUFF, and McDANIEL, *loc cit*

an agitation rate of 230 r.p.m. for a 75-gal. tank holding 200 liters of medium.

Stefaniak and coworkers[1] found an aeration rate of 1 volume of air per minute for each volume of medium in 100-gal. tanks holding 200 liters of medium to be optimum. Taylor[2] described a commercial process in which not less than 400 cu. ft. of air per minute were blown into the end of a fermentation tank of approximately 5,000-gal. capacity through a sparger.

Johnson[3] has discussed the influence of aeration on yields of penicillin in shaker flasks and tank fermentations. In Table 161 are shown some of the results obtained by him during a study of the effect of the rate of aeration on tank fermentations.

TABLE 161.—EFFECT OF AERATION RATE ON TANK FERMENTATION*†
(Culture X-1612 was grown on a medium containing 3 per cent lactose, 4 per cent steep liquor solids, and 1 per cent calcium carbonate)

Aeration, vol /min	Penicillin yield (70 hr.) units/ml.	CO_2†	Age at max NH_3 level, hr.
0 15	278	2.7	47
1 0	490	8.9	25
1 5	497	10.0	26

* JOHNSON, M. J., Ann. N. Y. Acad. Sci., 48 (Art. 2). 57 (1946).
† Volume of CO_2 per minute per 1,000 volumes of medium, average 20th to 60th hour

PRESSURE.—The use of pressure in tanks to increase the effectiveness of aeration is common practice. Stefaniak and his associates[4] usually carried out fermentations at a tank pressure of 20 lb. per sq. in. They found that reduction of the pressure to 2 lb. per sq. in. did not affect the fermentation adversely, but that a tank pressure of 40 lb. per sq. in. resulted in reduced yields of penicillin.

AGITATION.—The efficiency of aeration and yields are increased by effective agitation of the medium. Some data on agitation are presented in Table 157 which appears on page 732. A fermentation tank with aerating and agitation devices is shown in Fig. 99.

ANTIFOAM AGENTS.—A number of chemical agents have been suggested for use to prevent excessive foam formation during the production of penicillin by submerged-culture methods. Prevention of excessive foaming results in better yields of penicillin.

1 STEFANIAK, GAILEY, BROWN, and JOHNSON, loc. cit.
2 TAYLOR, T. H. M., Chem. Eng. Progress, 43 (No. 4): 155 (1947)
3 JOHNSON, loc. cit.
4 STEFANIAK, GAILEY, JARVIS, and JOHNSON, loc. cit.

Tributyl citrate was used as an antifoam agent by Foster and his coworkers.[1]

Stefaniak and his associates[1] found 3 per cent octadecanol dissolved in lard oil to be the most useful of the antifoam agents tested by them, which included lard, lard oil, 3 per cent octadecanol in lard oil, soybean
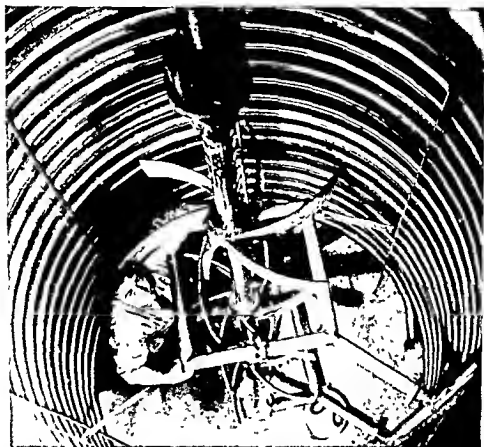
Fig 99 Interior view of a fermentor [*Courtesy of T H M Taylor, Chem Eng Prog, 43 (No 4) 155 (1947)*]

oil, 3 per cent octadecanol in soybean oil, vegifat Y, and Nopco defoamer. Lard, lard oil, and 3 per cent octadecanol in lard oil were found to be nontoxic in concentrations of 0 1 to 1 0 per cent. Although Nopco defoamer and vegifat Y were the most effective foam breakers, they were likewise the most toxic of the antifoam agents examined Three per cent octadecanol in soybean oil was toxic in shake-flask but not in tank fermentations.

[1] Foster, Woodruff, and McDaniel, *loc cit*
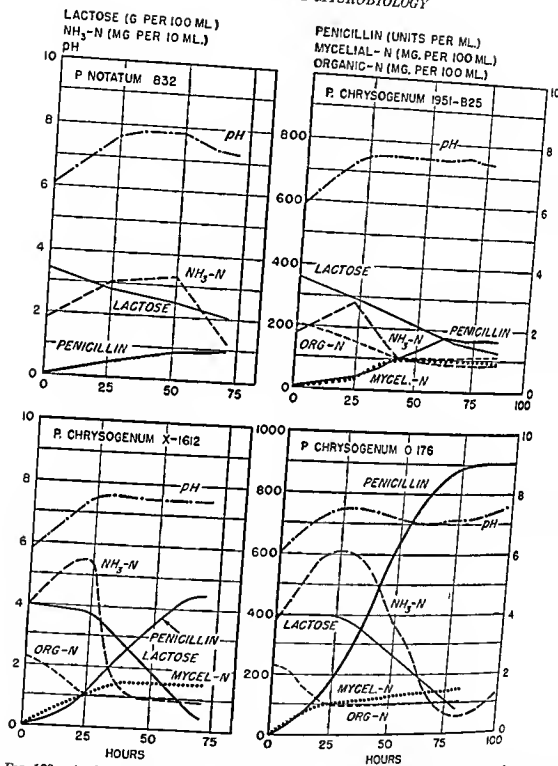
Fig. 100.—Analytical comparison of four *Penicillium* strains Medium: 3 to 4 per cent of lactose, 4 per cent of steep liquor solids, and 1 per cent of calcium carbonate [Courtesy of F. B. Gailey, J. J. Stefaniak, B. H. Olson, and M. J. Johnson, Jour. Bact., 52 (No. 1) 129 (1946).]

Antifoam agents, in general, reduced the pH levels of the fermentation media progressively as their concentrations were increased.

PREVENTION OF CONTAMINATION.—In no fermentation is it more essential to prevent contamination of the medium than in the penicillin fermentation Contamination usually produces rapid destruction of penicillin. In order to prevent contamination, it is necessary to sterilize all fermentation tanks, pipe lines, and equipment with which the penicillin may come into contact. Steam is the agent used for sterilization of facilities and media. Antifoam agents must be sterilized before aseptic addition to the medium Starters must be added aseptically. All air used for aeration must be sterilized

Biochemistry —Studies concerning the interrelationship of such factors as lactose consumption, penicillin production, pH, ammonia nitrogen, and mycelium weight have been made by Moyer and Coghill;[1][2] Foster, Woodruff, and McDaniel;[3] Stefaniak and associates;[4][5] Johnson;[6] and others. Figure 100 shows a comparison of biochemical changes induced by four different strains of penicillin-producing molds For a detailed discussion of this subject, the reader is referred to the original articles.

Yields —The data on yields obtained by Gailey and associates[7] by growing the molds in a medium containing 4 per cent glucose, 2 per cent corn-steep liquor solids, salts, and 0 5 per cent CaCO₃, and by using a production medium containing 4 per cent corn-steep liquor solids, 3 to 4 per cent lactose, and 1 per cent CaCO₃, are illustrative of those secured in submerged fermentation The fermentations were carried out in the tanks illustrated in Fig 95, each of which contained 200 liters of medium seeded with 10 per cent of inoculum The medium was stirred with an agitator revolving at the rate of 270 r p m and aerated with 200 liters of air per minute The pressure within the tanks was maintained at 20 lb per sq in A temperature of 23°C was used Table 162 shows the yields of penicillin obtained from different mold cultures

Recovery of Penicillin.—The object of the recovery process is to separate the penicillin from the mold protoplasm, the products of mold metabolism other than penicillin, and the unused portion of the medium During the process, the penicillin is concentrated and purified

[1] MOYER, A J, and R D COGHILL, *Jour Bact*, **51** (No 1) 57 (1946)
[2] *Ibid*, p 79
[3] FOSTER, WOODRUFF, and McDANIEL, *loc cit*
[4] STEFANIAK, GAILEY, JARVIS, and JOHNSON, *loc cit*
[5] STEFANIAK, GAILEY, BROWN, and JOHNSON, *loc cit*
[6] JOHNSON, *loc cit*
[7] GAILEY, F B, J J STEFANIAK, B H OLSON, and M J JOHNSON, *Jour Bact*, **52** (No 1) 129 (1946)

Since the unused portion of the medium contains sufficient nutrients to support the growth of bacteria and other microorganisms that might rapidly destroy the penicillin, great care must be exercised to prevent its contamination during the recovery process. The use of aseptic handling methods, refrigeration, and rapid handling are some of the ways of protecting the penicillin. Sometimes a disinfectant may be added to the medium to protect the penicillin.

TABLE 162.—SUMMARY OF PENICILLIN YIELDS IN CULTURE COMPARISON
EXPERIMENTS[1]
(Tank fermentations)

| Culture designation | No. fermentations | Penicillin yield | | Concurrent yield[a] with 1951.B25, units/ml |
		Average, units/ml	Best, units/ml.	
832	1		98	214
1951 B25	7	169	215	
25099	2	216	214	184
35217	1	.	275	245
35347	1		255	245
45417	2	199	229	245
15-U-1	3	157	200	214
R-38	2	109	135	165
R-1139	2	158	160	214
R-1201	2	147	148	214
R-1205	2	160	191	214
X-1612	46	369	558	245
Q176	6	761	901	
Q176-A8	6	286	360	

[1] GAILEY, F. B., J J STEFANIAK, B H OLSON, and M J. JOHNSON, Jour Bact, 52 (No 1): 129 (1946)
[a] Culture widely used in experimental studies and in early industrial fermentations Employed for control purposes

The first step in the recovery process is the removal of the mold mycelium by filtration or centrifuging. Continuous vacuum filters, Bird-Young filters, and basket centrifuges have been used for this purpose. The mold protoplasm thus separated is washed to save as much penicillin as possible.

There are a number of general methods used for concentrating penicillin and other antibacterial compounds. These include extraction with suitable organic solvents; adsorption on a suitable solid surface, such as activated carbon; low temperature evaporation; and precipitation. The

first two of these methods are the most common and the last two may be used in conjunction with them, according to Kavanagh.[1]

There are a number of methods for extracting penicillin and various modifications of these. In one method, described by Smith,[2] the broth is mixed with amyl acetate, or other suitable solvent, cooled, and agitated vigorously while the pH is adjusted to 2.0 to 2.5 with an acid such as phosphoric acid. The penicillin is transferred to the solvent as the acid. The mixture is centrifuged (after the addition of a surface-active agent to prevent sludge formation in the centrifuge due to protein precipitates). The solvent is next agitated with water while alkali is added to bring the pH to 7 to 7.5. The penicillin passes into the aqueous phase as the sodium salt. The solvent and aqueous phases are separated by centrifuging or by gravity. The penicillin may be taken up with another solvent and then extracted with a smaller amount of water containing sodium bicarbonate or sodium hydroxide if the sodium salt is desired; or with calcium carbonate or calcium hydroxide, if the calcium salt is desired.

Better purification of penicillin may be obtained by using solvents of different types in succession, according to Smith;[2] for example, amyl acetate, followed by chloroform. Occasionally a third solvent may be used.

Although a pH of 2 to 3 is most favorable for the extraction of penicillin in such solvents as amyl acetate, chloroform, and ether, the penicillin is very unstable at this pH. Therefore, the extraction must be made rapidly and at a low temperature.

In one large penicillin-production plant,[3] the process is essentially as follows: The mold mycelium is separated from the fermentation medium by filtration and washed to remove traces of penicillin. The mycelium-free broth and washings are mixed with activated carbon, usually about 2 to 2.5 per cent by weight, and the penicillin is adsorbed on it. The activated carbon is separated from the broth by means of filtration through a plate-and-frame filter press and is washed to remove the broth. The penicillin is eluted from the carbon with an 80 per cent acetone solution. A water-immiscible solvent is then used to remove the acetone from the solution. The aqueous phase, which contains the penicillin, is separated from the solvent phase by means of centrifuges. The solution of penicillin, which contains about 10 times as much penicillin as the original fermentation broth, is cooled to 0°C and adjusted to pH 2. The free acid of penicillin is next extracted with a water-immiscible solvent from the aqueous solution. The aqueous phase, which contains

[1] Kavanagh, F., *Advances in Enzymol.*, **7**: 461 (1947).
[2] Smith, *op. cit.*, p. 308.
[3] Anon, *De Laval Centrifugal Rev.*, **33** (No. 1): 8010 (1947).
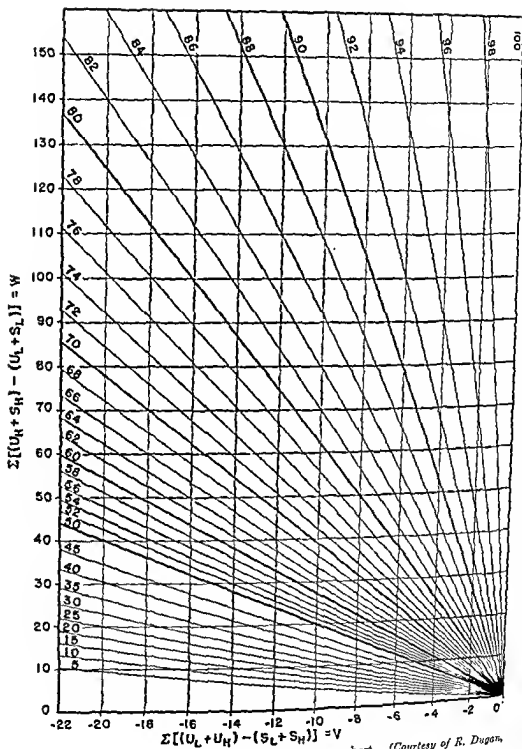
Fig. 101.—Penicillin assay chart. (Courtesy of R. Dugan.)

additional impurities, is separated from the water-immiscible solvent by means of centrifuges. A concentrated solution of sodium bicarbonate is added to the solvent containing the penicillin for the purpose of extracting the penicillin as its sodium salt. The salt is filtered to remove pyrogens and other precipitates. It is then dried from the frozen state under vacuum.

Another procedure for recovering penicillin is illustrated in Fig. 97.

Units of Penicillin.—According to Welch and associates,[1] the Oxford unit is that quantity of partially purified solution of arbitrary strength which, when diluted in a phosphate buffer saturated with ether, results in an inhibition zone of 24 mm. diameter when tested by the cup-plate method. For the series dilution method, the Oxford unit is the amount of penicillin that, when dissolved in 50 ml. of meat extract, just inhibits completely the growth of *Staphylococcus aureus*, the test strain. The international unit is the specific penicillin activity contained in 0.6 microgram of the primary standard, crystalline sodium penicillin G. One milligram of the International Standard is equivalent to 1,667 units of crystalline sodium penicillin G.

Testing and Assaying.—Since September, 1943, the Food and Drug Administration, Federal Security Agency, has tested each lot of commercial penicillin for potency, sterility, toxicity, pyrogenicity, moisture content, etc. Information concerning these tests and methods for assay for antibiotic drugs, including penicillin and compounds containing it, is published under Title 21—Food and Drugs, Chap. I—Food and Drug Administration, Part 141, in the Federal Register. The certification of batches of penicillin is considered in Part 146 of Chap. I.

The following Food and Drug Administration regulations for tests and methods of assay of penicillin have been published in the Federal Register:[2]

Section	Title
141 1	Sodium penicillin, calcium penicillin, potassium penicillin; potency
141 2	Sodium penicillin, calcium penicillin, potassium penicillin; sterility
141 3	.
141 4	.
141.5	Sodium penicillin, calcium penicillin, potassium penicillin

    (a) Moisture
    (b) pH
    (c) Clarity
    (d) Microscopical test for crystallinity of sodium penicillin and potassium penicillin
    (e) Stability of crystalline penicillin
    (f) Crystalline penicillin G
    (g) Penicillin K content

[1] WELCH, H., R. P. HERWICK, and A. C. HUNTER, *Jour. Am. Pharm. Assoc., Pract. Pharm. Ed.,* 6 (No. 4): 93 (1945).
[2] *Federal Register,* Apr. 4, 1947, et seq.

A study of the foregoing titles indicates the various types of penicillin products that are being marketed and the large number of tests and assays that are made by the Food and Drug Administration

A number of methods have been devised for assaying the potency of penicillin, some of which are referred to in Table 163 The most important of these methods is the cylinder-plate method, which was first described by Abraham and his associates [1] Schmidt and Moyer[2] refined this method, and Schmidt[3] further modified it. A device for placing the cylinders on the assay plates was developed and described by Reeves and Schmidt.[4] In modified form, the cylinder-plate method is the standard method of assay used by the Food and Drug Administration.[5]

Other biological methods exist for determining the potency of penicillin in broth concentrates, pure solutions, blood, and body fluids. Among these may be mentioned the turbidimetric method proposed by Fleming[6] and Abraham and associates[1] as an alternate method and

[1] Abraham, L. P., E. Chain, C. M. Fletcher, A. D. Gardner, N. G. Heatley, M. A. Jennings, and H. W. Florey, *Lancet*, 2 (No 6155 of 211) 177, 189 (1911).
[2] Schmidt, W. H., and J. Moyer, *Jour. Bact.* 47 (No 2) 199 (1911).
[3] Schmidt, W. H., *Bull. Health Organization of the League of Nations*, 12 (1 st No 9), (1911).
[4] Reeves, M. D., and W. H. Schmidt, *Jour. Bact.* 49 (No 4) 395 (1915).
[5] *Federal Register*, April 4, 1917, 13 F.R. 2215, 2217–2226, etc.
[6] Fleming, A., *Brit. Jour. Expt. Path.* 10: 226 (1929).

described by Foster[1] and others; the serial-dilution method described by Schmidt and Moyer;[2] and the experimental syphilis method of Rake and coworkers.[3]

Chemical and physical-chemical methods for separating, assaying, and analyzing penicillins have been developed, which include, amongst others, the chromatographic method of Martin and Synge;[4] the chemical-assay method for penicillin G, described by Sheehan and his coworkers;[5] the infrared analysis of penicillins, reported by Barnes and associates;[6] and the chemical assay method of Murtaugh and Levy.[7]

Improved methods for separating various types of penicillin have been developed by Fishbach and his associates,[8] and by Craig and fellow workers.[9]

Procedures have been devised for estimating the quantities of each of several different types of penicillin. One of the first of these was reported by Schmidt, Ward, and Coghill,[10] in which two test organisms were used. Buck and associates[11] devised an *in vivo* assay procedure in which mice were experimentally infected with *Borrelia novyi*. Later Higuchi and Peterson[12] described a method wherein three test organisms were used (*Staphylococcus aureus* 209-P, *Bacillus brevis*, and organism "E").

Donovick and collaborators[13] have carried out studies on the "quantitative differential analysis of mixtures of several essentially pure penicillin types."

A qualitative test for penicillin has been described by Kavanagh.[14]

[1] Foster, J W, *Jour Chem*, **144**: 285 (1942).

[2] Schmidt, W H, and A J Moyer, *Jour. Bact*, 47 (No 2): 199 (1944)

[3] Rake, G, W B Dunham, and R. Donovick, *Jour. Infectious Diseases*, 81: 122 (1947).

[4] Martin, A J P, and R L. M. Synge, *Biochem. Jour.*, 35: 1358 (1941).

[5] Sheehan, J C, W J Mader, and D J. Cram, *Jour. Am. Chem Soc*, 68: 2407 (1946)

[6] Barnes, R. B., R C Gore, E. F. Williams, S G. Linsley, and E Peterson, Paper presented at Conference on Antibiotic Research, Washington, D C., Jan. 31 and Feb. 1, 1947, under auspices of the Antibiotics Study Section, National Institute of Health

[7] Murtaugh, J, and G B. Levy, *Jour. Am. Chem Soc.*, 67: 1042 (1945).

[8] Fishbach, H., M Mundell, and T E. Eble, *Science*, 104: 84 (1946).

[9] Craig, L C, G H. Hogeboom, V DuVigneaud, and F H. Carpenter, Unpublished report distributed by the Antibiotics Study Section, National Institute of Health

[10] Schmidt, W. H, G E. Ward, and R D. Coghill, *Jour. Bact*, 49: 411 (1945)

[11] Buck, M, A. C Farr, and R J. Schnitzer, *Science*, 104 (No. 2703): 370 (1946).

[12] Higuchi, K, and W H. Peterson, *Ind. Eng Chem*, *Anal Ed*, 19: 68 (1947).

[13] Donovick, R, D Lapedes, and F Pansy, *Jour. Bact*, 54 (No. 4): 425 (1947)

[14] Kavanagh, F, *Science*, 105: 189 (1947).

TABLE 163—SOME METHODS FOR ASSAYING THE POTENCY OF PENICILLIN

Method	Media	Test organism	Reference
Cylinder (Food & Drug Administration)	Special nutrient agar pH 6.5 to 6.6 after sterilization	Staphylococcus IDA 209-P	Federal Register Apr. 4, 1947, 12, Federal Register 2215, 2217-2226 [1]
Cylinder plate	Special nutrient agar, (medium I of Schmidt and Moyer)	Staph. aureus IDA 209-P	Schmidt, W. H. and A. J. Moyer Jour. Bact. 47 (No. 2) 199-208 (1944); Reeves, M. D. and W. H. Schmidt Jour. Bact. 49 (No. 4) 395-400 (1945)
Cup plate or Oxford cylinder plate	Nutrient agar or modified nutrient agar	Staph. aureus	Abraham, E. P., E. Chain, C. M. Fletcher, A. D. Gardner, N. G. Heatley, M. A. Jennings, and H. W. Florey Lancet, 2 177-188, 149 (1941); Heatley N. G. Biochem. Jour. 38 61-65 (1944)
3-hr and 2-hr cylinder plate	Similar to those of Schmidt and Moyer (above)	Staph. aureus NRRL No. 313 or rough form of B. subtilis NRRL No. B-558	Pratt, R. and J. Dufrenoy Nature 159 576 (1947)
Broth dilution	Beef heart infusion broth containing 0.25 per cent glucose	Staph. aureus	Foster, J. W. and H. B. Woodruff Jour. Bact. 46, 187-202 (1943)
Serial dilution	Special nutrient broth (medium II of Schmidt and Moyer)	Staph. aureus 209-P	Schmidt, W. H. and A. J. Moyer Jour. Bact. 47 199-208 (1944)
Serial dilution	Broth used by IDA in assaying penicillin	Bacillus subtilis NRRL	Randall W. A., C. W. Price, and H. Welch Science 101 (No. 2627) 363 (1945)
Turbidimetric	Double-strength nutrient broth	Staph. aureus (Oxford strain)	Foster, J. W. Jour. Bact. Chem. 144 285-296 (1942)
Tissue culture	Bartlett broth containing 10 per cent serum	Diplococcus pneumoniae Type 7	Heilman D. H. Am. Jour. M. Sc. 207 157 (1944)
Experimental syphilis	Rat fetus	Treponema pallidum	Rake G., W. F. Dunham and H. Bunnagh Jour. Infectious Diseases 81 123 (1947)

[1] Amendments are published from time to time in the Federal Register.

Limitations in methods used in testing antibiotic substances have been discussed by Welch, Randall and Knudsen, and others.

Cylinder-Plate Method.—This method has been widely used for determining the potency of penicillin and, as described below, is the standard test used by the Food and Drug Administration[1] for sodium, calcium, and potassium penicillins.

The test, in brief, consists of adding 21 ml. of a nutrient agar to a sterile petri dish of greater depth than that ordinarily used for bacteriological studies, allowing the agar to harden, adding 4 ml. of a second type of nutrient agar which has been seeded with the test organism, spreading the seeded agar uniformly over the surface of the base agar, permitting it to solidify, dropping four sterile cylinders onto the agar, filling the cylinders with suitable dilutions of the working standard of penicillin and of the sample of penicillin being assayed, incubating the plates at 37°C. for 16 to 18 hr., measuring the diameters of the zones of inhibition, making certain calculations with the data obtained, and determining the potency by reference to special charts.

The materials required for the test are the test organism, three kinds of media, petri dishes, porcelain covers for the petri dishes, cylinders (cups), and suitable dilutions of the working standard of penicillin and of the sample of penicillin being tested.

TEST ORGANISM.—The test organism is a special strain of *Staphylococcus aureus*, designated as F.D.A. 209-P or No. 9144 of the American Type Culture Collection, Washington, D.C.

CULTURE MEDIA.—The ingredients used in the preparation of the culture media must meet the requirements of the U.S.P. or N.F. However, the media may be prepared from dehydrated mixtures and distilled water, provided that they have the same composition when reconstituted. Likewise, "minor modification of the individual ingredients are permissible if the resulting media possess growth promoting properties at least equal to the media described."[2]

A nutrient agar medium of the following composition is used for carrying the test organism and for the seed layer, which may be designated as medium 1:

Peptone	6 0 g
Pancreatic digest of casein	4.0 g
Yeast extract	3 0 g.
Beef extract	1 5 g.
Glucose	1 0 g
Agar	15 0 g
Distilled water, q s	1,000.0 ml.
pH 6 5 to 6 6 after sterilization	

[1] Food and Drug Administration, *Federal Register*, Apr. 4, 1947.
[2] *Ibid.*

A nutrient agar medium of simpler composition, which may be designated as medium 2, is used for the base layer:

Peptone	6 0 g.
Yeast extract	3 0 g.
Beef extract	1 5 g.
Agar	15 0 g
Distilled water, q s	1,000 0 ml
pH 6 5 to 6 6 after sterilization	

A nutrient broth medium, which is used for the preparation of an inoculum of the test organism and which may be designated as medium 3, has the following composition.

Peptone .	5 0 g.
Yeast extract	1 5 g.
Beef extract	1 5 g.
Sodium chloride	3 5 g.
Glucose	1 0 g.
Dipotassium phosphate	3 68 g
Potassium dihydrogen phosphate	1 32 g
Distilled water, q s	1,000 0 ml
pH 7 0 after sterilization	

PETRI DISHES.—These should measure 20 by 100 mm. Porcelain covers, which are glazed on the exterior, are used for the test

CYLINDERS (CUPS).—The cylinders are made from stainless steel and have the following measurements outside diameter, 8 mm (±0.1 mm), inside diameter, 6 mm (±0.1 mm), and length, 10 mm. (±0.1 mm)

THE WORKING STANDARD.—The working standard of penicillin is obtained from the Food and Drug Administration. It is kept in tightly stoppered vials, which are stored in larger stoppered tubes containing anhydrous calcium sulphate, and refrigerated at 15°C (59°F.) or at a lower temperature constantly From 4 to 5 mg of the working standard are weighed out in an atmosphere containing 50 per cent or less of relative humidity and used to make up a stock solution of the desired concentration. Sterile 1 per cent phosphate buffer at a pH of 6 0 is used for diluting the penicillin The stock solution is stored at a temperature of about 10°C for 1 day only Working dilutions are prepared from the stock solution.

PREPARATION OF PLATES.—This consists of adding 21-ml portions of uninoculated nutrient agar (medium 2) to sterile petri dishes, allowing it to solidify, adding 1 ml of seeded agar to the surface of the base agar in each plate, and dropping the cylinders onto the seeded agar

The seeded agar is prepared as follows The test organism, which is carried on slants containing medium 1 and transferred about once a week, is used to inoculate nutrient broth (medium 3) The broth culture is

incubated at 37°C. for 16 to 24 hr. Two milliliters of this culture are mixed thoroughly with a 100-ml. portion of the agar (medium 1), which has been melted and cooled to 48°C. Four milliliters of the seeded agar are placed on the surface of the base agar in each petri dish and spread uniformly by tilting the dish back and forth.

A suspension of the test organism, which may be used over a period of a week, may be prepared instead of the broth culture for seeding the agar. The test organism is grown on an agar slant (medium 1) at 37°C. for 24 hr. The growth is washed from the surface with 2 ml. of sterile physiological saline solution and transferred to a large surface of agar, for example that provided by 300 ml. of agar in a Roux bottle. It is uniformly distributed over the entire surface of the agar by means of sterile-glass beads. The seeded agar is incubated at 37°C. for 24 hr. and at room temperature for 24 hr. The growth on the surface of the agar is suspended in 50 ml of sterile physiological saline. The suspension thus obtained is standardized "by determining the dilution which will permit 20 per cent light transmission through a filter at 6500 angstrom units in a photoelectric colorimeter."[1] Each 100-ml portion of medium 1 is inoculated with 1.5 to 2.0 ml. of the standardized suspension. Four milliliters of the seeded agar are added to each petri dish containing 21 ml. of base agar, as described above.

The stainless-steel cylinders are sterilized in separate containers, cooled, and aseptically added to the surface of the seeded agar by dropping them from a height of 0.5 in. with a mechanical guide. Four cylinders are added to each plate, separated by about 90° intervals on a radius of 28 cm. The petri dishes have porcelain covers, glazed on the top.

PREPARATION OF THE SAMPLE.—A stock solution of the sample being assayed may be prepared by dissolving it in sterile distilled water.

ASSAY.—A total of four petri dishes, each containing four cylinders, are used for each sample. Two cylinders in each plate are reserved for dilutions of the working standard and two for dilutions of the sample. One cylinder in each plate is filled with a dilution of the working standard that contains 1.0 unit per ml. of penicillin. A second cylinder in each plate is filled with a dilution of the working standard that contains 0.25 unit per ml. A third cylinder in each plate is filled with a dilution of the sample that contains an estimated 1.0 unit per ml. A fourth cylinder in each plate is filled with a dilution of the sample that contains an estimated 0.25 unit per ml. The plates are placed very carefully in the incubator spaces and stored for 16 to 18 hr. at 37°C. After the incubation period, the diameters of the zones of inhibition are measured to the nearest 0.5 mm, using a suitable measuring device such

[1] *Ibid.*

as a colony counter containing a measuring scale (in millimeters) etched into the glass that supports the dish over the light source.

ESTIMATION OF POTENCY AND ERROR —In order to determine the potency of the sample, certain values must be calculated from the assay data and used in connection with the chart shown in Fig. 101. The values required are designated as V and W. These values may be determined by adding together all the v values and the w values, respectively, for all the plates The v value for each plate may be determined by the following formula: $v = (U_L + U_H) - (S_L + S_H)$. The w value for each plate may be determined by the following formula.

$$w = (U_H + S_H) - (U_L + S_L)$$

S_H and S_L represent the diameters (in millimeters) of the zones of inhibition of the 1.0-unit and the 0 25-unit dilutions of the standard, respectively, and U_H and U_L, the diameters of the zones of inhibition of the corresponding dilutions of the test sample After the w and v values have been calculated, the point on the chart (Fig. 101) that corresponds to these values is located and the potency ascertained from the radial lines.

The chart should not be used for determining the potency of a sample wherein the potency is lower than 50 per cent or higher than 150 per cent of the standard Where the potency varies to this extent, the assay should be repeated, using a higher or lower dilution of the sample

For details concerning the procedure to be followed in estimating the error of assay, the reader is referred to Part 141, Chap. 1—Food and Drug Administration, Title 21—Food and Drugs

Serial-dilution Method —This method may be used to assay the penicillin in culture liquor or in concentrated ethereal solutions and is simple and fast, according to Schmidt and Moyer [1] In brief, it consists of preparing an inoculum of the test organism; filtering a sample of penicillin, if this is necessary, preparing dilutions of the penicillin with culture medium seeded with the test organism; incubating the tubes; reading the results, and calculating the potency in terms of units

The test organism, *Staphylococcus aureus* F D A 209-P, is grown at 37°C in a medium of the following composition, which is medium II of Schmidt and Moyer.[1]

Peptone (Bacto)	5 0 g
Yeast extract (Bacto)	1 5 g
Beef extract (Bacto)	1 5 g
Glucose, hydrated commercial corn sugar	1 0 g
Sodium chloride	3 5 g
1 per cent phosphate buffer pH 7 8	500 ml
Distilled water to make	1 000 ml

[1] Schmidt and Moyer, *loc cit*

The 1 per cent phosphate buffer is made up by dissolving 2.63 g. of monopotassium phosphate (KH_2PO_4) and 7.36 g. of dipotassium hydrogen phosphate (K_2HPO_4) in distilled water and adjusting the final volume to 1 liter. Schmidt and Moyer[1] advise heat-sterilizing the buffer to prevent microbial contamination and saturating the solution with toluene when it is employed to dilute solutions of penicillin.

When the test organism has incubated for 20 hr. in broth medium described above, 1 ml. of the broth culture is used to inoculate 200 ml. of the same medium that has been sterilized and cooled in a 500-ml Erlenmeyer flask. The seeded medium is used as the diluent in preparing dilutions of the penicillin in a series of sterile tubes.

The penicillin sample is rendered free of bacteria by passing it through a Seitz or sintered-glass (grade 5 on 3) filter, according to Schmidt and Moyer.[1]

Dilutions of the penicillin sample in the broth containing *Staphylococcus aureus* are made as follows: One milliliter of the sample of penicillin is mixed with 9 ml. of the broth to give a 1:10 dilution. One milliliter of the 1:10 dilution is mixed in turn with 9 ml. of broth to give a 1:100 dilution. One-milliliter portions of the 1:100 dilution of the sample mixed with 3-, 4-, 5-, 6-, and 7-ml. portions of the broth yield dilutions of 1:400, 1:500, 1:600, 1:700, and 1:800, respectively. One-half-milliliter portions of the 1:100 dilution of the sample mixed with 4-, 4.5-, 5-, and 5.5-ml portions of the broth yield 1:900, 1:1,000, 1:1,100 and 1:1,200 dilutions of the sample, respectively.

The contents of the tubes are thoroughly mixed and then the tubes are incubated at 37°C for 18 or 40 hr.

When the tubes are observed at the end of 18 hr., the reciprocal of the highest dilution of the penicillin sample which prevents growth of *Staphylococcus aureus* in that time is multiplied by the factor 0.045, which is the approximate number of Oxford units of penicillin required to inhibit the test organism. For example, if a dilution of 1:1,100 prevents growth of the test organism but a dilution of 1:1,200 does not, there are $1,100 \times 0.045 = 49.5$ Oxford units per ml of sample.

When the tubes are observed at the end of 40 hr. (which Schmidt and Moyer recommend as the better time, for the results are more definite and easier to read), the factor is 0.1 (the approximate number of Oxford units per milliliter required to inhibit growth of the test organism). Thus, if a dilution of 1:500 prevents growth of the test organism but a 1:600 dilution does not, there are $500 \times 0.1 = 50$ Oxford units per ml of sample.

¹ *Ibid.*

Some Uses in Medicine.—Penicillin is used by members of the medical profession for the prevention and treatment of infections caused by staphylococci, streptococci, pneumococci, gonococci, and certain other Gram-positive organisms.

TABLE 164.—SOME PENICILLIN PRODUCTS, POTENCIES, AND MANNERS OF USE

Product	Potency	Manners of use
Crystalline penicillin G (sodium or potassium)	100,000/vial 200,000/vial 500,000/vial 1,000,000/vial	In sterile isotonic sodium chloride solution (or in pyrogen-free sterile distilled water or in sterile 5 per cent dextrose solution) intravenously, intramuscularly, or subcutaneously
Crystalline penicillin G (calcium or sodium) in oil and beeswax (Romansky formula)	300,000 units/ml of oil-beeswax mixture	Intramuscularly (never intravenously)
Crystalline penicillin G (sodium) in nebulized normal saline	200,000 units	Inhalation
Buffered crystalline penicillin G (sodium or potassium) tablets	50,000 units each 100,000 units each	Orally
Crystalline penicillin G troches	5,000 units/troche	Orally
Penicillin ointment dermatologic	1,000 units/g	Topically and locally
Penicillin ointment ophthalmic	1,000 units/g	Locally

According to Keefer,[1] the use of penicillin is indicated for staphylococcic infections, such as bacterial endocarditis, carbuncles, infections dermatitis, empyema, furunculosis, meningitis, osteomyelitis (acute or chronic), pneumonia, wounds, and burns, for streptococcic infections, such as cellulitis, endocarditis, mastoiditis, peritonitis and puerperal sepsis, for pneumococcic infections, such as empyema, meningitis and pneumonia, particularly sulphonamide-resistant cases, for malignant edema or

[1] KEEFER, C. A., *Jour. Am. Med. Assoc.*, **122:** 1217 (1943).

gas gangrene caused by clostria, such as *Cl. welchii* and *Cl. sporogenes;* and for gonococcic infections, anthrax, vincent's infections, erysipeloid, and other infections caused by penicillin-susceptible organisms. Penicillin is indicated for use in preventing secondary infections which may follow tooth extractions, tonsillectomies, or other operations. Penicillin has proved helpful in the treatment of actinomycosis, diphtheria, and syphilis, but further study is necessary in order to fully evaluate these uses.

Penicillin is ineffective against Gram-negative bacillary infections, such as typhoid fever, dysentery, undulant fever and tularemia; *Mycobacterium tuberculosis; Hemophlus influenzae* and *H. pertussis; Pasteurella pestis; Vibrio comma; Kbebsiella pneumoniae; Monilia albicans; Trichomonas vaginalis;* etc.

STREPTOMYCIN

Historical.—Streptomycin, which was named after the genus of antinomycetes (*Streptomyces*) that produces aerial mycelium and sporulates, was discovered in 1943 by Waksman and his associates at the N.J. Agricultural Experiment Station, Rutgers University.[1] The finding of this chemotherapeutic agent culminated extensive surveys and numerous analyses and tests, all part of a search for an antibiotic that would be effective against Gram-negative bacteria, bacteriostatically, bactericidally, *in vitro, in vivo,* and in the presence of body fluids, and that would be relatively nontoxic to the cells and tissues of the body.

Types of Organisms against Which Effective.—Streptomycin is particularly destructive of Gram-negative organisms, for example, *Escherichia coli, Aerobacter aerogenes, Pseudomonas aeruginosa, Shigella gallinarum,* and *Serratia marcescens.* Urinary infections and bacteremias due to Gram-negative bacteria, meningitis, and other infections caused by *Hemophilus influenzae* and especially tularemia, are some of the diseases that may be treated successfully with this antibiotic.[2,3] Some promise is held in connection with the treatment of tuberculosis, where streptomycin exerts a slow but positive effect against the development of the disease, which is caused by *Mycobacterium tuberculosis.* Gram-positive sporeforming bacteria, such as *Bacillus subtilis* and *B. cereus,* and certain strains of *Staphylococcus aureus,* are susceptible to the influence of this antibiotic.

Table 165 supplies information on the sensitivity of some microorganisms to streptomycin [4]

[1] Schatz, A., E. Bugie, and S A. Waksman, *Proc. Soc. Expt. Biol. Med.,* **55:** 66 (1944).

[2] Nicols, D. R., and W. E. Herrell, *Jour. Am. Med. Assoc.,* **132** (No. 4): 200 (1946).

[3] Committee on Chemotherapeutics and Other Agents, National Research Council, *Jour. Am. Med. Assoc.,* **132** (No. 1): 4–11 (1946); **132;** (No. 2): 70–77 (1946).

[4] Waksman, S. A., and A. Schatz, *Jour. Am. Pharm. Assoc.,* **34:** 273 (1945).

TABLE 165.—RANGE IN SENSITIVITY OF GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA AND ACTINOMYCETES TO THE BACTERIOSTATIC ACTION OF STREPTOMYCIN[1][2]

Gram-negative organisms	Micrograms per cubic centimeter	
Aerobacter aerogenes	0 5	61 0
Bacillus anthracis	0 375	
Brucella abortus..	0.5	3 75
B. melitensis .	0.5	
B. suis	0 5	
Eberthella typhi	1 0	37 5
Erysipelothrix muriseptica	2 5	
Escherichia coli	0 3	3 75*
E. communior	1 0	4 0
Hemophilus influenzae	1 56	5 0
H pertussis	1 25	3 0
Klebsiella ozogenes	0 375	1 5
K pneumoniae	0 625	256
Malleomyces mallei	10 0	>10 0
Neisseria gonorrheae	5 0	
N. intracellularis	5 0	
Pasteurella lepiseptica	0 5	2 5
P pestis	0 75	1 5
P tularensis	0 15	0 3
Proteus vulgaris	0 4	3 2
Pseudamonas aeruginosa[3]	2 5	25 0
Salmonella aertrycke	4 0	10 0
S enteritidis	0 5	
S. schottmülleri	2 0	
S suipestifer	60 0	
Shigella paradysenteriae	0 25	3 75
Vibrio comma	6 0	37 5
Gram-positive organisms		
Actinomyces bovis	3 75	
Clostridium butyricum	8 31	
Cl septicum	>10[4]	
Cl sordelli	>10[4]	
Cl tetani	>10[4]	
Cl welchii	>10[4]	
Corynebacterium diphtheriae	0 375	3 75
Diplococcus pneumoniae	8 0	
M tuberculosis, var hominis	0 15	
Staphylococcus aureus[5]	0 5	>16 0
Streptococcus fecalis	50 0	
Strept hemolyticus	2 0	>16 0
Strept lactis	4 0	
Strept salivarius	5 0	25 0
Strept viridans	>16	120

[1] The Committee on Chemotherapeutic and Other Agents, National Research Council *Jour Am Med Assoc* 132 4-11 (Sept 7) 70 77 (Sept 14 1946)
[2] Micrograms of streptomycin per cubic centimeter of suitable medium required to inhibit growth.
[3] A group of *Ps aeruginosa* and *Ps fluorescens* (by Hirsch[1]) some req ired over 256 micrograms per cu cm to inhibit growth
[4] Some staphylococci have been reported (by Hirsch[1]) to require over 256 micrograms per cu cm to inhibit growth
[5] Some colon bacilli have been reported to require 800 to 20,000 micrograms per cu cm. to inhibit growth

Structure.—Streptomycin has the chemical formula $C_{21}H_{39}N_7O_{12}$. It appears to consist of streptidine linked glycosidally to streptobiosamine.[1] When it is hydrolyzed in an aqueous acid solution, streptidine ($C_8H_{18}N_6O_4$), an optically inactive hydroxylated base and one of the stereoisomeric forms of 1,3-diguanido- 2,4,5,6-tetrahydroxycyclohexane, is formed.[2] Streptidine has the following structural formula.[3]
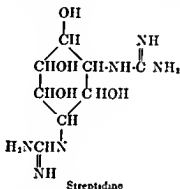
Streptidine

When streptomycin hydrochloride is treated with methanol containing hydrogen chloride it is degraded to streptidine and a streptobiosamine derivative.[1] When the streptobiosamine derivative is subjected to acid hydrolysis, N-methyl-l-glucosamine is produced:[4]

N-Methyl-l-glucosamine

Kuehl, Jr, and his associates[5] have proposed the following structural formula for streptomycin, in which streptobiosamine is attached at the number 4 carbon atom of streptidine:

[1] BRINK, N. G., F. A. KUEHL, JR., and K. FOLKERS, *Science*, 102:506 (1945).

[2] PECK, R. L., R. P. GRABER, A WALTI, E. W PEEL, C. E HOFFHINE, JR., and K. FOLKERS, *Jour. Am. Chem Soc*, 68:29 (1946).

[3] PECK, R. L., C. E. HOFFHINE, JR, E W. PEEL, R P. GRABER, F. W. HOLLY, R MONINGO, and K. FOLKERS, *Jour. Am. Chem. Soc*, 68:776 (1946).

[4] KUEHL, E. H., JR, E H FLYNN, F W. HOLLY, R. MONINGO, and K. FOLKERS, *Jour. Am. Chem. Soc*, 68:536 (1946).

[5] KUEHL, F A, JR, R L. PECK, C E HOFFHINE, JR, E. W. PEEL, and K. FOLKERS, *Jour. Am. Chem Soc*, 69:1234 (1947)
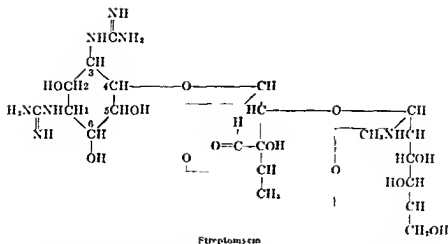
Streptomycin

Properties of Streptomycin.—Streptomycin is soluble in water and insoluble in acetone, chloroform, and ether. It is readily adsorbed on charcoal from alkaline solutions and may be eluted with dilute acid solutions. Streptomycin is relatively stable. According to Regna, Wasselle, and Solomons,[1] there was no loss of potency in commercial samples that were stored at room temperature for 12 months and that contained less than 1 per cent of moisture. They also reported that there was no loss in potency over a period of 60 days in aqueous samples of streptomycin at a pH of 3 to 7 and stored at or below 28°C. However, a 50 per cent reduction in activity resulted from storage of a streptomycin solution at a pH of 5.5 and at a temperature of 93°C for 37 hr. According to Waksman and Schatz,[2] less than 50 per cent of the activity of streptomycin was destroyed by heating it at 100°C for 10 min. Oswald and Nielsen[3] reported that solutions containing 100 and 1,000 units per ml of streptomycin at pH 6.0, 7.0, and 8.0 and stored at 10°C were stable for 3 months.

Strong alkaline solutions are destructive to streptomycin, the action being very rapid when the solutions are boiled.

Streptomycin is highly resistant to the action of enzymes and other biological agents.

Crude and highly concentrated solid preparations are hygroscopic.

Toxicity.—Streptomycin is relatively nontoxic to man. Extensive studies concerning the toxicity of streptomycin have been carried out by Molitor,[4] by Molitor and associates,[5] by Robinson,[6] by Brown and Hin-

[1] Regna, P. P., L. A. Wasselle, and J. A. Solomons, *Jour. Biol. Chem.*, 165: 631 (1946).
[2] Waksman and Schatz, *loc. cit.*
[3] Oswald, E. J., and J. K. Nielsen, *Science*, 105: 184 (1947).
[4] Molitor, H., *Ann. N. Y. Acad. Sci.*, 48 (Art. 2): 161 (1946).
[5] Molitor, H., *et al. Jour. Pharmacol. Expl. Therap.*, 86: 151 (1946).
[6] Robinson, H. J., *Ann. N. Y. Acad. Sci.*, 48 (Art. 2): 119 (1946).

shaw,[1] and by others. The subject has been reviewed by Hirshfeld and Buggs,[2] by Murray, Paine, and Finland,[3] and by others.

Among the types of reactions observed after the use of streptomycin are the following: (1) the presence of pain, irritation, or tenderness at the site of infection; (2) histamine-like reactions, as for example, headache, fever, flushing of the skin, and blood pressure drop; (3) disturbances of the eighth cranial nerve, such as *transient deafness*; (4) *sensitization manifestations* (skin eruption, etc.); and (5) casts and albumin in the urine. The first two of these reactions have been observed most generally in connection with the use of earlier impure products. Engels[4] has reported that the impurity producing the histamine-like reactions has been eliminated by an extraction process. *The third type of reaction usually follows prolonged treatment.* Casts and albumin ordinarily disappear rapidly after the treatment is stopped. *The most serious of the reactions, according to Murray, Paine, and Finland,[5] is the disturbance of the vestibular function, usually after prolonged treatment with large quantities of streptomycin. The severity of this reaction appears to be less with the purer preparations*

Clinical Uses.—The clinical uses of streptomycin have been reviewed by Keefer and others;[5] Paine, Murray, and Finland;[6] Hirshfeld and Buggs;[7] Wenner;[8] and others.

Production.—*Streptomycin is produced commercially by submerged-culture methods.* However, it may be produced by surface-culture methods, particularly on a laboratory basis. Production consists of growing the organism on a suitable medium under carefully controlled conditions.

Organisms Used.—The organisms used for streptomycin production are strains of *Streptomyces griseus*, an actinomycetes that produces aerial mycelium and spores *Strept. griseus* was isolated at Rutgers and was similar morphologically, biochemically, and culturally to one isolated in the same laboratory 28 years earlier, according to Waksman and Schatz[9]

[1] BROWN, H A, and R. C. HINSHAW, *Proc Staff Meetings Mayo Clinic*, 21: 347 (Sept. 4, 1946).

[2] HIRSHFELD, J W, and C. W. BUGGS, *N Y. State Jour Med.*, 47 (No. 11): 1276 (1947).

[3] MURRAY, R, T. F. PAINE, and M. FINLAND, *New Eng Jour. Med*, 236: 70 (May 8, 1947)

[4] ENGELS, W H, *Chem Eng News*, 26 (No. 18). 1284 (1948).

[5] Committee on Chemotherapeutics and Other Agents, op. cit, p. 70

[6] PAINE, T F., R. MURRAY, and M FINLAND, *New Eng. Jour Med*, 236: 748 (May 15, 1947)

[7] HIRSHFELD and BUGGS, loc. cit.

[8] WENNER, H A, *Jour. Kansas Med Soc*, June, 1947.

[9] WAKSMAN and SCHATZ, loc. cit

Although the strains of *Strept. griseus* that produce streptomycin are not always absolutely stable, the danger of strain degeneration is very small.[1] Waksman and Schatz stated that vegetative growth (without spores) should not be used for inoculation purposes, since the antibiotic is not always produced under such conditions.
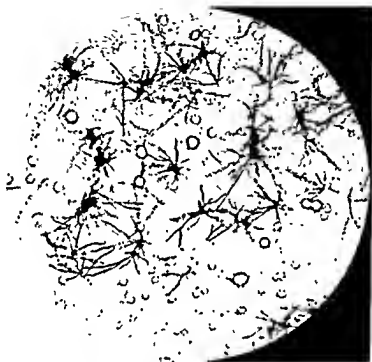
Fig 102 *Streptomyces griseus* streptomycin-producing strain   (*Courtesy of S A Waksman, New Jersey Agricultural Experiment Station, New Brunswick*)

The streptomycin-producing qualities of *Strept griseus* have been improved by strain selection and by irradiation with ultraviolet light, according to Stanley[2]

Raw Materials —The medium used for the manufacture of streptomycin should contain the ingredients essential for the proper growth of *Strept griseus* and for the production and accumulation of streptomycin and should be relatively free from histamine-like substances

Waksman and Schatz[1] found that *Strept griseus* produced the highest yields of streptomycin in a medium containing meat extract   This ingre-

[1] WAKSMAN and SCHATZ, *loc cit*
[2] STANLEY, A R, *Jour Bact*, **53**: 254 (1947)

dient could be replaced with corn-steep liquor, but the use of the latter increased the difficulties encountered in purifying the antibiotic. The medium devised by Waksman and Schatz and used in early commercial production[1] had the following composition:

Glucose	. · . . . . 10 g.
Peptone	. . . . 5 g.
Meat extract	. . . . 5 g.
NaCl	. . . . 5 g
Tap water	. · . . . . 1,000 ml.
Final pH	. . . . 6 5-7.0

It was discovered that both the nitrogen and carbon sources were non-specific; that is, glucose and peptone could be substituted with other substances. For example, sodium nitrate, tryptones, amino acids, and both hydrolyzed and unhydrolyzed casein were about as effective as peptone as sources of nitrogen; whereas starch and glycerol were satisfactory substitutes for glucose.

Table 166 supplies information on the influences of the nitrogen source and meat extract on the production of streptomycin in stationary cultures.

TABLE 166 —INFLUENCE OF NITROGEN SOURCE AND MEAT EXTRACT ON THE PRODUCTION OF STREPTOMYCIN IN STATIONARY CULTURES[1,2]

Nitrogen source	Meat extract, g./liter	Days of incubation					
		7		9		14	
		μ	pH	μ	pH	μ	pH
Sodium nitrate[3]	0	0	5 2	0		0	5 4
Sodium nitrate	0 5	2	6 7	3	6 8	1	6 9
Sodium nitrate	5 0	24	7 4	65	7 7	52	7 9
Peptone[4]	0	4	7 9	4	8 1	2	8 0
Peptone	0 5	14	7 6	25	7 8	14	7 8*
Peptone	5 0	37	7 5	75	8 0	60	8 3
Glycine[4]	0	0	6 0	0 .		0	5 9
Glycine	0 5	58	7 7	26	7.8	17	8.3
Glycine	5 0	75	8 0	85	8 4	28	8.6

[1] WAKSMAN, S A., and A. SCHATZ, *Jour Am Pharm Assoc.*, **34**: 273 (1945).
[2] Activity expressed in terms of micrograms of pure streptomycin per milliliter of medium
[3] Two grams per liter
[4] Five grams per liter.

Rake and Donovick[2] have studied the nutritional requirements of *Strept. griseus* and formulated media, containing neither beef extract nor

[1] PORTER, R. W., *Merck Rept.*, July, 1947.
[2] RAKE, G., and R. DONOVICK, *Jour. Bact.*, **52**: 223 (1946).

corn steep liquor, which produced as high as 250 units of streptomycin per milliliter and from which, it was reported, the antibiotic could be recovered more readily in a purified state The media devised by Rake and Donovick contained soybean meal as a source of nitrogen and other nutrients. Salt was found to be an essential constituent for producing satisfactory yields of streptomycin

TABLE 167—STREPTOMYCIN PRODUCTION IN VARIOUS MEDIA[1]

Medium No	Constituents %				No of replicate flasks	Vol of medium per flask, ml		Days of incubation				
	Soybean Meal[3] %	Glucose	Beef extract	Sodium chloride				3	4	5	6	7
1	1 5	1 0			2	100	pH	6 8	7 0	6 9	7 5	7 8
							u/ml	1 0		5 71	5 7	9 8
2*	1 5	1 0	0 5	0 5	4	200	pH	7 0	7 1	7 4	7 2	7 4
							u/ml	6 1	10 7	27 2	37 4	73 2
3	1 5	1 0	0 1		4	100	pH	7 3	7 8	8 2	8 3	8 5
							u/ml	37 1	44 1	96 2	121 0	114 0
4	1 5	1 0	0 1	0 5	6	100	pH	7 1	7 7	8 2	8 4	
							u/ml	123 5	147 0	146 0	156 0	
5	1 5	1 0	0 2	0 5	5	100	pH	6 8	7 7	8 2	9 0	
							u/ml	69 2	156 0	160 0		
6*	1 5	1 0	0 5		7	100	pH	7 2	7 9	8 3	8 6	
							u/ml	41 5	100 0	168 0	181 0	
7	1 5	1 0		0 5	16	100	pH	7 1	7 6	8 1	8 3	
							u/ml	120 3	170 0	187 5	212 0	
8	1 0	1 0		0 5	6	100	pH	7 0	7 1	7 4	7 9	8 3
							u/ml	129 0	148 0	201 0	236 0	237 0

[1] RAKE, G., and D. DONOVICK Jour Bact 57 223 (1946)
[2] Made up in distilled water
[3] The soybean meal employed contained from 41 to 44 per cent protein
[4] Units of streptomycin per milliliter of broth
* Media 2 and 6 are the same except that 200 ml of medium was dispensed per flask. This medium (No. 6 in the table) differs from the medium recommended by Waksman and Schatz in that the peptone and meat extract of the latter has been replaced with soybean meal

Table 167 summarizes data from the researches of Rake and Donovick [1] The media tested were dispensed in 500-ml Erlenmeyer flasks in 100-ml amounts, except in the case indicated After sterilization, the contents of each flask were inoculated with 0 5 ml of a spore suspension This was prepared by suspending the spores of *Strept griseus*, grown on Kramsky's asparagine gluco-agar, in distilled water and shaking them for 30 min with glass beads The flasks were incubated at 24°C on a shaking machine

An examination of the table indicates that the best media for streptomycin production (numbers 7 and 8) contained soybean meal, glucose

[1] *Ibid.*

and sodium chloride. **Actually the highest yields of the antibiotic were obtained when the percentages of these ingredients were 1, 1, and 0 5, respectively.**

Bennett[1] reported that the following raw materials gave comparable yields of streptomycin on a laboratory scale: corn-steep water, soybean flour, some peptones, acid hydrolyzed casein, acid hydrolyzed rabbit fur, acid hydrolyzed wheat gluten, acid hydrolyzed stillage obtained from the yeast-alcohol fermentation of wheat mash, and asparagus butt juice

Corn-steep water is a rich source of histamine-like substances, 400 to 1,000 micrograms of such ingredients per gram having been found in some samples of steep water, according to Stanley.[2]

In an effort to determine what ingredients in steep water were responsible for the stimulation of streptomycin production, various quantities of corn-steep ash were added to a medium containing glucose and an enzymatic digest of casein. The effects of various quantities of corn-steep ash on streptomycin production are shown in Table 168.

TABLE 168.—SUBSTITUTION OF CORN-STEEP ASH FOR CORN-STEEP WATER IN STREPTO- MYCIN PRODUCTION MEDIA[1]

Amount of Corn-steep Ash, G./100 Ml	Highest Average Streptomycin Titer, Micrograms/Ml.
0 25	430
0 12	430
0 06	650
0 03	500
0 015	175
0 007	70
0	40

[1] STANLEY, A R Paper presented before Ohio-Michigan Regional A C S Meeting in Toledo, Ohio, on Mar. 15, 1947

An examination of Table 168 indicates the marked influence of corn-steep ash on streptomycin production. Under the conditions of the fermentation, the highest yield was obtained when the medium contained a concentration of 0.06 g. per 100 ml. of corn-steep ash.

In view of the fact that about 70 per cent of the corn-steep ash is composed of potassium and phosphorous, potassium phosphate was substituted for the ash in the medium and comparable results were obtained, according to Stanley[2] and Bennett.[3] Potassium chloride may also be used instead of corn-steep ash to stimulate streptomycin production. Stanley reported that it was preferred to potassium phosphate since it did not have such a narrow range of optimum concentration.

[1] BENNETT, R. E., *Jour. Bact.*, **53**: 254 (1947).
[2] STANLEY, A R., Paper presented Mar 15, 1947.
[3] BENNETT, R. E, *Jour. Bact.*, **53**: 254 (1947)

Stanley and Bennett reported that beef extract also contained histamine-like substances in large amounts.

Commercial Production.—The production of streptomycin on a commercial scale has been described by Silcox,[1] Porter,[2,3] Kirkpatrick,[4] Engels,[5] and others.
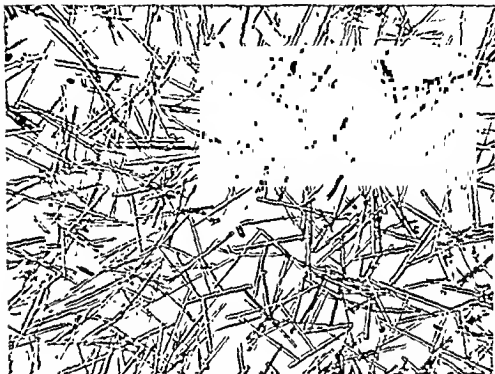
FIG. 193. Crystalline streptomycin trihydrochloride-calcium chloride double salt. *(Photo from Merck & Co. Inc.)*

HISTORICAL.—The first concern to manufacture streptomycin industrially was Merck and Company, which was in operation by the spring of 1946. Streptomycin hydrochloride and streptomycin sulphate were produced initially but they could not be as highly purified as was desired. However, in 1945, Peck and coworkers[6] reported the preparation of a crystalline double salt of streptomycin trihydrochloride and calcium chlo-

[1] SILCOX, H., *Chem. Eng. News*, **24**: 2763 (1946)

[2] PORTER, R. W., *Chem. Eng.*, **53**: 91 (1946)

[3] PORTER, R. W., *Merck Rept.*, July, 1947

[4] KIRKPATRICK, S. D., *Chem. Eng.*, **54** (No. 11): 91 102 (1947)

[5] ENGELS, W. H., Paper presented at A.A.A.S. Symposium on Antibiotics, Chicago, Dec. 29 30, 1947

[6] PECK, R. L., N. G. BRINK, F. A. KUEHL, JR., E. H. FLYNN, A. WALTI, and K. FOLKERS, *Jour. Am. Chem. Soc.*, **67**: 1866 (1945)
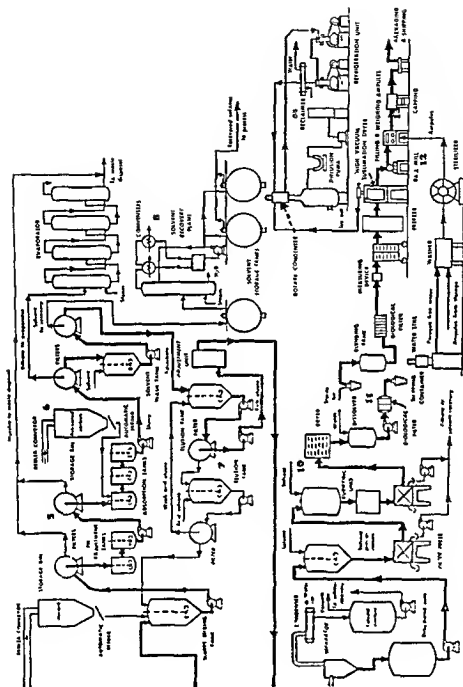
Fig. 101.—Flow sheet of streptomycin production [*Courtesy of the Editor, Chem. Eng., 53 (No. 10), 91 (1946).*]

ride (refer to Fig. 103) which was of high purity. This salt has the formula $C_{21}H_{39}O_{12}N_7 \cdot 3HCl - 1\frac{1}{2}CaCl_2$. Since July, 1947, Merck and Company has manufactured the crystalline calcium chloride salt of streptomycin exclusively.[1,2]

Fig. 105.—Partial view of fermentation unit in plant of Merck & Co., Inc., at Elkton, Va. (Photo from Merck & Co., Inc.)

Figure 104 presents a flow sheet, outlining streptomycin production. It will be realized that changes in certain parts of the process have taken place since the original publication, particularly in the medium and final stages of extraction.

FERMENTATION.—The raw materials (usually a hydrolyzed protein-containing substance, a carbohydrate, and salts) are mixed with water in a mixing tank at about 10 times the concentration in which they will be used and pumped to a storage tank. From the storage tank, the concentrate is pumped into each of a series of four fermenters of increasing sizes

[1] PORTER, R. W., Merck Rept., July, 1947

[2] KIRKPATRICK, S. D., Chem. Eng., 54 (No. 11): 91-102 (1947).

as required. Water is added to dilute the nutrient medium to the desired concentration. The fermentation medium is then sterilized at 120°C., cooled, and inoculated with a starter. The fermentation is carried out at 25 to 30°C with aeration and agitation for the required length of time, the contents of each fermenter serving as the inoculum for the next larger fermenter. The fourth fermenter is of 15,000-gal. capacity (Fig. 104).
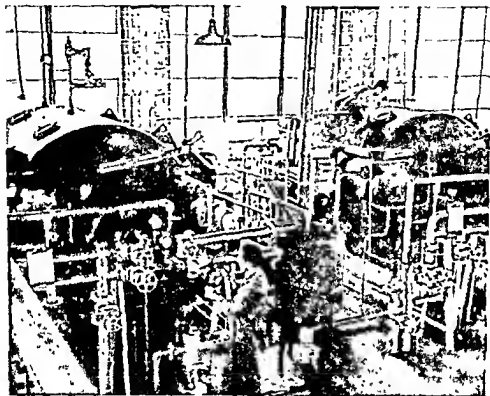
Fig. 106. Filtration units used to remove mycelium from the fermented solutions. *(Photo from Merck & Co., Inc.)*

The fermenters (refer to Fig. 105) are constructed of carbon steel and are equipped with agitators, air spargers, cooling coils or jackets, heating coils, viewing glasses, etc. Valves, stuffing boxes, sight glasses, and all joints and connections are protected with steam seals. Pipe lines are maintained under steam pressure when not in use.

Aeration is supplied by blowing sterile air through the fermentation medium and by agitation. According to Porter, about 0.03 cu. ft. of air per minute per gallon of fermenter capacity is passed through the filters for sterilization. The rate of aeration and agitation must be controlled to prevent foaming.

It may be necessary to use an antifoam agent, if excessive foaming

occurs. Such material must be sterilized before use and must not be toxic to *Strept. griscus* or the antibiotic and must not interfere with the subsequent recovery process.

The fermentation, which is carried out at 25 to 30°C., is considered completed when a maximum yield of streptomycin has been obtained. The fermented broth is withdrawn to a storage tank immediately. From this point on, the process becomes continuous.

Fig. 107 —Extraction units
acidified alcohol and filtered
resistant alloys prevent corrosion during the stream
Inc.)

FILTRATION.—The fermented broth is filtered to remove the mycelium. This is accomplished in Oliver precoated[1] pressure filters (Fig 106), which have high filtration rates. Before the broth goes to the filter, filter aid (nonadsorbing) is mixed with it in a slurry mixing tank, the amount added depending upon the rate of flow of the liquor. The admixture flows through the filter which has been precoated with filter aid. After this primary filtration, the pH of the filtrate is adjusted and then it is polished by passage through a second filter. The filter cakes are discarded

[1] ENGELS, *op cit*

ANTIBIOTICS

ADSORPTION ON ACTIVATED CARBON.—Activated carbon is mixed with
the clear broth in a series of three adsorption tanks. The streptomycin
and some of the impurities are extracted from the broth by adsorption on
the activated carbon, which is then automatically fed to a pressure filter,
where the spent broth is separated out. The adsorbate is washed on the
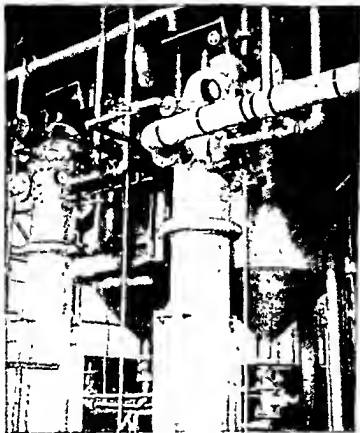
Fig. 108. Evaporators for concentrating crude streptomycin solutions. (*Photo from Merck
& Co., Inc.*)

filter with dilute alcohol for the purpose of removing impurities soluble in
alcohol.

ELUTION.—The streptomycin is eluted from the activated carbon with
dilute alcoholic hydrochloric acid by a two-stage countercurrent process,
according to Porter.[1] It is removed as the trihydrochloride, while impu-
rities remain adsorbed on the activated carbon. The trihydrochloride is
separated from the activated carbon by filtration.

The elution units are especially constructed in order to prevent the
formation of metallic salts that would contaminate the streptomycin.

[1] Porter, R. W., *Chem. Eng.*, **53** (No. 10) 91 (1946).

To prevent contamination of the product, the workers must use special cleaning techniques and change into sterile uniforms and shoes before going to the processing areas.

In order to reduce the numbers of microorganisms in the air, three procedures are followed: all the air entering the building is passed through

FIG. 111 — Weighing streptomycin powder This procedure is carried out on delicate prescription balances in sterile cubicles The entire room is air-conditioned with filtered air and "sterility" is maintained through the use of ultraviolet ray lamps (Photo from Merck & Co., Inc.)

special filters, triethylene glycol is used as a disinfecting aerosol, and ultraviolet lamps are used in the cubicles where the antibiotic is exposed to the air for a short time.

Yields.—Stanley[1] has reported that yields of 600 to 800 micrograms of streptomycin per milliliter were not unusual from a strain of *Strept. griseus* obtained as a result of irradiation with ultraviolet light, and that a yield of over 900 micrograms per ml. in a shake-flask fermentation was obtained with one strain.

[1] STANLEY, A. R., Paper presented before Ohio-Michigan Regional A.C.S. Meeting in Toledo, Ohio, on Mar. 15, 1947.

Metabolism of Strept. griseus.—Waksman, Schatz, and Reilly[1] have supplied significant information concerning the metabolism and chemical nature of *Strept. griseus* Their studies included growth of the organism in both stationary and submerged cultures.

The stock medium used in the investigations contained 5 g each of peptone, meat extract, and sodium chloride, and 10 g of glucose per liter. In the research carried out with stationary cultures, 250-ml. portions of the medium were placed in 1-liter Erlenmeyer flasks, sterilized, cooled, and inoculated with spore suspensions of *Strept. griseus* The seeded flasks were incubated at 28°C. The weights observed were those obtained by filtering the mycelium and spores on weighed papers, drying at 65°C., and weighing to secure the increase in weight due to the dried material.

An examination of Tables 169 and 170 which are concerned with stationary cultures indicates that *Strept. griseus* attained a maximum amount of growth in 10 days and declined slowly thereafter. The amino nitrogen in the culture broth reached a high level in 5 to 7 days. The production of streptomycin was accelerated after the seventh day when the amino-nitrogen content was high

In the case of submerged cultures, the maximum amount of growth was obtained usually in 3 to 5 days, followed by lysis of the mycelium. Streptomycin production and sugar consumption were rapid between the second and fifth days (reference is made to Table 171). The pH of the filtrate increased, particularly during the first two days.

TABLE 169—RATE OF GROWTH AND STREPTOMYCIN PRODUCTION OF *Strept. griseus* IN STATIONARY CULTURES
(Per 250-ml portions of medium)

Incubation, days	Growth, g	Streptomycin, units ml
4	0 364	<5
5	0 437	8
7	0 449	13
10	0 695	128
15	0 640	140
21	0 597	125

[1] WAKSMAN S A, SCHATZ and H C REILLY *Jour Bact* 51 753 1946

Gottlieb and Anderson,[2] in a study involving morphological and physiological factors in streptomycin production in shake cultures, observed

[1] WAKSMAN, S. A., SCHATZ, and H C REILLY *Jour Bact*, 51: 753 (1946)
[2] GOTTLIEB, D, and H W ANDERSON *Bull Torrey Botan Club* 74 No 4 293 302 (1947)

that the peak of streptomycin production followed the peak of growth of *Strept. griseus*, that the abilities of different strains of *Strept. griseus* to increase the pH of the medium were not correlated with their abilities to produce streptomycin, and that synthesis of streptomycin occurred only when oxygen was present.

TABLE 170.—NITROGEN FRACTIONS IN *Strept. griseus* CULTURES[1]
(Per 250-ml portions of medium)

Incubation, days	Nitrogen in mycelium		NH₂-N in broth, mg	NH₂-N in broth, mg.	Total N[2] accounted for, mg.	Ash content, per cent
	Per cent	Mg				
0			4 3	35 3		
4	10 0	35 2	22.6	57.3	115 1	12 1
5	9 7	40 3	37 8	69.5	147 6	16 4
7	10 0	55.8	55 0	73 3	185 0	14 6
10	8 9	62 4	63 3	66 8	192 5	13 9
15	0 6	55 2	92 6	79.3	227 1	10 0
21	7 2	37 6	95 1	70.8	203 5	11 0

[1] WAKSMAN, S A., A SCHATZ, and H C REILLY, *Jour Bact.*, 51:753 (1945)
[2] The original broth contained 250.0 mg of total nitrogen

TABLE 171.—RATE OF GROWTH OF *Strept. griseus* AND STREPTOMYCIN PRODUCTION IN SHAKEN CULTURES[1]

Incubation, days	Growth, g	pH of filtrate	Residual glucose, mg /ml	Streptomycin, units/ml
0		6 8	10 2	0
1	0 018	6 9	9 3	5
2	0 237	8 5	7 6	5
3	0 394	8 6	5 6	63
5	0 370	8 4	0 5	84
7	0 248	8.7	0 5	62
10	0 140	8 9	0 5	51

[1] WAKSMAN, S A., A SCHATZ and H C REILLY, *Jour Bact.*, 51. 753 (1945)

Extraction and Purification of Streptomycin in the Laboratory.—After the maximum amount of streptomycin has been produced, action is taken to isolate it from the culture medium. The growth material (chiefly mycelium) of *Strept. griseus* is separated from the liquid medium by centrifuging or by filtering.[1] Since streptomycin is adsorbed on certain types of filters and filter aids, only those which are known not to adsorb it should be used. An active charcoal, for example Norit, is added to the liquid

[1] WAKSMAN and SCHATZ, *loc. cit.*

medium, which has been freed from cellular material, to adsorb the strep-
tomycin. The charcoal containing the adsorbed streptomycin is sepa-
rated from the liquid medium by centrifuging or filtering and is washed
with alcohol to remove impurities. It is next washed with acid-alcohol
to elute the streptomycin; afterwards, it is separated out. The acid-
alcohol solution of streptomycin is neutralized and filtered to remove pre-
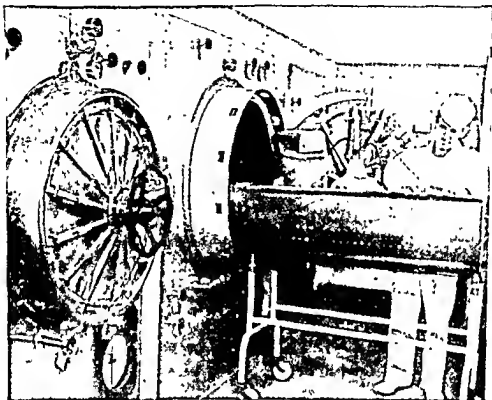
Fig. 112. Autoclaves. All containers used in the finishing of streptomycin are sterilized.
(Photo from Merck & Co. Inc.)

cipitated impurities. Ten volumes of ether (in which streptomycin is
insoluble) are added which take up the alcohol but not the streptomycin.
The antibiotic is left as an aqueous concentrate (yellow, brown, or red).
By drying the aqueous concentrate under vacuum or by precipitating it
with acetone, a preparation of solid streptomycin may be obtained. In
an alternate method, a concentrate may also be obtained by drying the
neutralized acid eluate under vacuum.[1]

Further purification may be obtained by precipitating the acid eluate
obtained from the charcoal adsorbate (refer to the foregoing paragraph)

[1] KUHL, F. A., JR., R. L. PECK, A. WALTI, and K. FOLKERS, *Science*, 102: 34
(1945).

with phosphotungstic acid[1,2] and treating as follows. The bases liberated from the phosphotungstate are converted to a crude picrate which is fractionated by chromatographic methods. Picric acid is removed and there remain one or more fractions which yield water-soluble and highly active amorphous products. Crystalline precipitates result when Reinecke salt, ammonium tetrathiocyano-diammono-chromate, $NH_4[Cr(SCN)_4(NH_3)_2]$, is added to the aqueous solutions of the active fractions. Pure streptomycin reineckate may be isolated by fractional crystallization. This insoluble reineckate may be rendered soluble by conversion to the hydrochloride or to sulphate salts

It is also possible to crystallize streptomycin as streptomycin-helianthate.[1] Methyl orange (helianthine B, or sodium p-dimethyl-aminoazobenzenesulphonate) is added to the acid eluate obtained from the charcoal adsorbate described above A relatively insoluble streptomycin-helianthate crystallizes from the solution. Soluble salts may be obtained by further treatment.[3] For example, by treating streptomycin-helianthate with a mixture of methyl alcohol and hydrochloric acid, streptomycin hydrochloride is formed. The liberated helianthine is removed and the hydrochloride is precipitated from the filtrate with ether. Streptomycin hydrochloride is a white powder.

Standardization.—The unit of streptomycin used in early reports was defined as "the amount of material which will inhibit the growth of a particular strain of *E. coli* in 1 ml. of nutrient broth or other suitable medium."[4]

Waksman[5] stated that the use of the foregoing unit proved satisfactory for production and isolation studies and for pharmacological investigations of streptomycin. However, he believed that this unit was unsuited for clinical use since the large number of units required created the impression that large doses were essential for effective chemotherapeutic purposes. He proposed the establishment of the following units'

(1) An S unit, or that amount of material which will inhibit the growth of a standard strain of *E. coli* in 1 ml. of nutrient broth or other suitable medium. This unit would thus correspond to the original *E. coli* unit.

(2) An L unit, or that amount of material which will inhibit the growth of a standard strain of *E. coli* in 1 liter of medium. An L unit is thus equivalent to 1,000 S units.

(3) A G unit, comparable to one gram of the crystalline material.

[1] WAKSMAN, SCHATZ, loc. cit.
[2] FRIED, J , and O. WINTERSTEINER, Science, 101: 613 (1945).
[3] KUEHL, PECK, WALTI, and FOLKERS, loc. cit.
[4] SCHATZ, A , E. BUGIE, and S. A. WAKSMAN, Proc. Soc. Expt. Biol. Med , 55: 66 (1944)
[5] WAKSMAN, S. A., Science, 102: 40 (1945).

Methods of Assay.—A comparatively large number of methods have been devised and used for the assay of streptomycin. These have been reviewed by Waksman and Schatz;[1] Murray, Paine, and Finland;[2] Hirshfeld and Buggs;[3] Wenner;[4] and others The review by Murray and associates[2] is particularly significant.

Biological and chemical methods of assay have been employed but the biological methods are the most suitable at the present time. Among the biological methods are the cup method of Stebbins and Robinson,[5] which has been extensively used, with and without modification; the paper disc-plate method of Loo and associates;[6] the broth-dilution methods of Donovick and his coworkers[7] and of Price, Nielsen, and Welch;[8] the slide-cell method of Heilman for estimating the amount of streptomycin in body fluids;[9] the turbidimetric method of Osgood and Graham;[10] and the streak-plate method of Waksman and Reilly [11] Among the chemical methods are those of Schenck and Spielman;[12] Scudi, Boxer, and Jelinek,[13] and Levy, Schwed, and Sackett[14]

The test organism used depends upon the method and purpose of assay. Among those which have been used are special strains of *Staphylococcus aureus* and *Bacillus subtilis* in the agar-diffusion assays, *Klebsiella pneumoniae*, *Staph. aureus* and *B. circulans* in broth-dilution methods; and *B. megatherium* in the slide-cell method

In carrying out assays, it is important to make sure that the medium will not contain substances which inhibit the antibacterial activities of streptomycin (refer to the section that follows) Likewise, it is essential that the conditions used be such that they may be readily duplicated in other laboratories.

The broth-dilution method, which was developed by Donovick,

[1] WAKSMAN, S A., and A. SCHATZ, *Jour. Am. Pharm Assoc*, **34**: 273 (1945)

[2] MURRAY, PAINE, and FINLAND, *loc cit*

[3] HIRSHFELD and BUGGS, *loc. cit*.

[4] WENNER, H. A., *Jour Kansas Med Soc*, June, 1947

[5] STEBBINS, R. B., and H J ROBINSON, *Proc Soc Expt Biol Med*, **59**: 255 (1945)

[6] LOO, Y. H., P. S. SKELL, H. H THORNBERRY, J EHRLICH, J M McGUIRE, G. M. SAVAGE, and J. C SYLVESTER, *Jour Bact*, **50** (No 6) 701 (1945)

[7] DONOVICK, R., D. HAMRE, F KAVANAGH, and G RAKE, *Jour Bact*, **50**: 623 (1945).

[8] PRICE, C. W., J. K. NIELSEN, and H WELCH, *Science*, **103**: 56 (1946)

[9] HEILMAN, D. H., *Proc Staff Meet Mayo Clinic*, **20**: 145 (May 16, 1945)

[10] OSGOOD, E E., and S M. GRAHAM, *Am Jour Clin Path*, **17**: 93 (1947)

[11] WAKSMAN, S A., and H C REILLY, *Ind Eng Chem., Anal Ed*, **17**: 556 (1945)

[12] SCHENCK, J.R., and M A SPIELMAN, *Jour Am Chem Soc*, **67**: 2276 (1945)

[13] SCUDI, J. V., G. L. BOXER, and V C JELINEK, *Science*, **104**: 486 (1946)

[14] LEVY, G B., P. SCHWED, and J W SACKETT, *Jour Am Chem Soc*, **68**: 528 (1946).

Hamre, Kavanagh, and Rake [illegible]
thricin, is described herein. [illegible]
method is more sensitive thr[illegible]
Woodruff. Solutions of antibiotics tested by this procedure must be
sterile and contain approximately 1.0 to 3.0 units of the antibiotic per ml

The test is carried out under standard conditions. The test organism, *Klebsiella pneumoniae*, is grown for 6 hr. at 37°C. in Difco yeast broth. A 1×10^{-6} dilution of the culture is made in 1 per cent tryptone broth at pH 7.2. Two-milliliter quantities of the seeded tryptone broth are placed aseptically into clear sterile glass tubes measuring 12×100 mm. These are stored in an ice box at 4°C.

Measured quantities of a standard solution and of the unknown solution of streptomycin (or streptothricin) are placed into the series of tubes prepared as described above, using acid-cleaned sterile 0.2-ml. Kahn pipettes. The standard solution of streptomycin contains 1.85 units per ml At least four tubes are used to determine the activity of the standard solution, which serves as a base for estimating the activity of the unknown solution. The unknown solution is prepared in such a manner that the concentration of streptomycin lies between 1.0 and 3.5 units per ml. Portions of the unknown solution varying in volume (0.10, 0.088, 0.077, 0.068, 0.059, 0.052, 0.046, 0 040, 0.035, 0.030 ml.) are added to the series of tubes containing *K. pneumoniae*. The assay with the dilutions of the unknown may be carried out in duplicate. If for any reason the test is not carried out at once, the tubes should be stored at 4°C.

The tubes containing the test organism and dilutions of streptomycin are placed in a 37°C. incubator for 15 to 17 hr., after which time observations are made for the presence or absence of growth.

Great care is exercised in making observations on the degree of growth in the tubes after the incubation period. Each tube is vigorously agitated and then held before a bright source of light (daylight lamp preferred). The absence of growth is reported by the use of the minus sign $(-)$; a trace, by the plus-or-minus sign $(\pm)$, and full or almost full growth, by the plus sign $(+)$. The end point is the minimum volume of the antibiotic solution that causes complete inhibition of *K. pneumoniae*

The following equation is used in computing the value of the unknown:

$$\frac{U_x}{U_e} = \frac{V_e}{V_x}; \quad \text{thus,} \quad U_x = \frac{U_e \times V_e}{V_x}$$

In this equation, U_e = the units per milliliter of streptomycin in the standard solution, U_x = the units per milliliter of streptomycin in the unknown, V_e = the volume of the standard solution required to cause

[1] DONOVICK, HAMRE, KAVANAGH, and RAKE, *loc. cit.*

complete inhibition of growth, and $U_x =$ the volume of the unknown solution required to cause complete inhibition of growth.

Inactivation of Streptomycin.—Knowledge concerning the chemical agents or conditions that cause inactivation or neutralization of the effects of streptomycin is of great importance, for it aids one (1) to use the antibiotic more intelligently and effectively, (2) to select the ingredients for the media used in testing its potency more accurately, and (3) to devise suitable tests for the determination of the sterility of the antibiotic.

CHEMICAL AGENTS —The effect of various chemical substances on the antibacterial activity of streptomycin *in vitro* has been studied by Geiger and associates;[1] Denkelwater, Cook, and Tishler;[2] Brink, Kuehl, and Folkers;[3] Donovick and Rake;[4] Bondi, Dietz, and Spaulding;[5] and others. The subject has been reviewed by Murray, Paine, and Finland.[6] Cysteine and β-mercaptoethylamine (sulphhydryl compound) inhibited the action of streptomycin against *B. subtilis.*[1,2] Cysteine reduced its action towards *E. coli* in a similar manner.[5] Hydrazine (0.002 M), hydroxylamine (0.002 M), semicarbazide (0.002 M), phenylhydrazine (0.002 M), and methylphenylhydrazine (0.001 M) (ketone reagents) likewise reduced the efficacy of the action of streptomycin against either *B. subtilis, E. coli,* or both.[1,2] Cevitamic acid (in a concentration of 0.4 mg. per liter) seriously reduced the effectiveness of the action of streptomycin against *E. coli, A. aerogenes, Staph. aureus,* and *B. subtilis.*[?] The chemical agent 2-aminoethanediol has also inactivated streptomycin. Bondi, Dietz, and Spaulding[5] have reported that the bacteriostatic activity of streptomycin for *E. coli* is lowered by the presence of reducing agents, such as cysteine, sodium thioglycollate, stannous chloride, sodium bisulphite, sodium hydrosulphite, sodium formate, and sodium thiosulphate. They also demonstrated that the antibacterial activity of streptomycin in infusion agar plates containing *E. coli* was reduced under anaerobic conditions.

UNFAVORABLE pH ACTION —The use of a sugar, such as glucose, levulose, or sucrose, medium which may be fermented by *B. subtilis, E. coli,* or other organisms with the production of sufficient acid to reduce the pH, has usually resulted in diminished activity on the part of streptomycin.[?] Hence the reduced antibacterial activity of the antibiotic in the presence of certain sugars is due in part at least to the production of an unfavorable

[1] GEIGER, W. B., J. B. GREEN, and S. A. WAKSMAN, *Proc. Soc. Expt. Biol. Med.,* 61 187 1946.

[2] DENKELWATER, R., M. COOK, and M. TISHLER, *Science,* 101 12 (1945).

[3] BRINK, KUEHL, and FOLKERS.

[4] DONOVICK, R., and G. RAKE, *Proc. Soc. Expt. Biol. Med.,* 61: 221 (1946).

[5] BONDI, A., JR., C. A. DIETZ, and F. H. SPAULDING, *Science,* 103: 399 (1946).

[6] MURRAY, PAINE, and FINLAND, *loc. cit.*

[7] GEIGER, GREEN, and WAKSMAN, *loc. cit.*

pH as the result of the formation of acid. Carbohydrates that serve as a source of carbon but that are not fermented with the production of sufficient acid to produce a harmful change in the pH have in general little or no effect on the antibacterial activity of streptomycin.

ANAEROBIC CONDITIONS.—According to Geiger, Green, and Waksman.[1] the production of anaerobic conditions through the use of hydrogen, nitrogen, or carbon dioxide gases has resulted in considerably reduced antibacterial activity by streptomycin against such facultative anaerobes as *E. coli, A. aerogenes, Proteus vulgaris,* and *Serratia marcescens.* The lowering of the pH of the medium under anaerobic conditions is believed to be a factor that is at least partly responsible for the reduced activity of the streptomycin.[1]

AGE OF CULTURE.—*Actively growing young cultures of bacteria are apparently more susceptible to streptomycin than older ones* (24 to 48 hr old) where the growth rate has decreased.

Donovick and Rake[2] have found that increasing the quantity of tryptone from 0.5 to 1 per cent in a medium containing only tryptone and water resulted in raising the minimum inhibiting concentration of streptomycin against *Klebsiella pneumoniae.* Likewise, the addition of glucose to the tryptone media increased the minimum inhibiting concentrations still further, but to a smaller degree.

Comparing the activity of streptomycin against *K. pneumoniae* in a broth containing 0.75 per cent tryptone with that in a liquid medium containing sodium thioglycollate (cystine, 0.75 g.; NaCl, 2.5 g.; glucose, 5 g.; agar, 0.75 g.; yeast extract, 5.0 g.; pancreatic digest of casein, 15.0 g.; sodium thioglycollate, 0.5 g.; resazurin (0.1 per cent), 1 ml.; and water to make 1,000 ml.), Donovick and Rake discovered that the minimum inhibiting concentration of streptomycin was considerably greater in the medium containing sodium thioglycollate than in the broth. The inhibitory effect of sodium thioglycollate decreased as the age of the medium increased. Sodium glycollate was also inhibiting but less so than sodium thioglycollate, Donovick and Rake suggested that the interfering action of sodium thioglycollate might be due to its effect in reducing the oxidation-reduction potential of the medium.

Streptomycin B.—This compound was isolated from streptomycin concentrates by Fried and Titus.[3] Some biological characteristics of it have been described by Rake and his associates.[4] According to the latter,

[1] GEIGER, GREEN, and WAKSMAN, *loc. cit.*
[2] DONOVICK and RAKE, *loc. cit.*
[3] FRIED, J., and E. TITUS, *Jour. Biol. Chem.,* 168: 391 (1947).
[4] RAKE, G., C. M. MCKEE, F. E. PANSY, and R. DONOVICK, *Proc. Soc. Expt. Biol. Med.,* 65: 107 (1947)

streptomycin B was less active *in vitro* on a weight basis than streptomycin against eight strains of bacteria. However, on a weight basis, both have approximately equal LD$_{50}$ values, based on studies with Swiss albino mice. In the case of experimental infections with *M. tuberculosis* (Ravenel strain), streptomycin B was about one-third as active as streptomycin on a weight basis and of about equal activity on a unit basis.

Dihydrostreptomycin.—Peck, Hoffhine, Jr., and Folkers,[4] reported on the formation of this compound by the catalytic hydrogenation of streptomycin.

BACITRACIN

Bacitracin was discovered and described by Johnson, Anker, and Meleney.[2] It is the name given to the antibiotic substance (or substances) produced by the Tracy I strain of *Bacillus subtilis*. The Tracy I strain was isolated from infected tissue removed during an operation on the compound fracture of the tibia of a patient.

Bacitracin is of particular interest because it has been effective in clearing infections resistant to penicillin.

Organisms against Which Effective.—Bacitracin is effective against Gram-positive bacteria principally,[2] for example, the aerobic and anaerobic streptococci, staphylococci, and micrococci, corynebacteria, and clostridia *in vitro*.[3] It is also effective against gonococci and meningococci, Gram-negative organisms, and spirochetes.[3] *In vivo*, it is effective against streptococcus, staphylococcus, and other types of infections,[4] and gas gangrene in guinea pigs.[5]

Chemical Properties.—Under this heading will be discussed solubilities, precipitability, stability, and other factors.

Solubilities.—Bacitracin is soluble in water, cyclohexanol, ethanol, isopropanol, methanol, and n-butanol. It is slightly soluble in cyclohexanone.[5] It is insoluble in acetone, benzene, chloroform, ether, and ethyl acetate.[6]

Precipitability.—Bacitracin is precipitated from water solutions or concentrates by certain metallic ions, organic acids, and other sub-

[1] Peck, R. L., C. L. Hoffhine, Jr., and K. Folkers, *Jour. Am. Chem. Soc.*, 68: 1390 (1946).

[2] Johnson, B. A., H. Anker, and F. L. Meleney, *Science*, 102 (No. 2650) 376 (1915).

[3] Miller, J. L., M. H. Slatkin, and B. A. Johnson, *Jour. Investigative Dermatol.*, 10 (No. 3) 179 (1918).

[4] Meleney, F. L., and B. Johnson, *Jour. Am. Med. Assoc.*, 133: 675 (1917).

[5] Johnson, B. H., H. Anker, J. V. Scudi, and J. Goldberg, In Vivo and In Vitro Laboratory Observations on Bacitracin, Special Report.

[6] Anker, H. S., B. A. Johnson, J. Goldberg, and F. L. Meleney, *Jour. Bact.*, 85 (No. 2) 249 (1918).

stances.[1,2] It is precipitated by the ions of heavy metals, those low in
the electromotive series causing inactivation of the antibiotic. Ions high
in the electromotive series, such as magnesium and zinc, do not inactivate
bacitracin. Magnesium oxide is used in the purification of bacitracin,
since the magnesium ion does not precipitate the antibiotic and since it
removes carbohydrates and other impurities from bacitracin preparations.

The antibiotic is precipitated from concentrates by azobenzene-p-
sulphonic, benzoic, furoic, salicylic, tannic, and trichloroacetic acids
Several of the acids cause a loss in the activity of the preparation. How-
ever, salicylic acid apparently does not inactivate bacitracin, according
to Anker and his associates.[1]

Bacitracin may be precipitated from water solution by high concen-
trations of sodium chloride, by acetone, by ammonium rhodanilate, by
molybdic acid, and by Reinecke's salt.[1,2]

Adsorption.—The antibiotic may be adsorbed on aluminum oxide,
charcoal, and Lloyd's reagent.[1,2]

Filtration.—Bacitracin may be filtered through a Berkefeld,[3] Cham-
berland,[3] or Seitz[3] filter.

Stability.—The dried, powdered product is fairly stable. According
to the Food and Drug Administration tentative specifications,[4] the expira-
tion date of vials of bacitracin is 12 months after the time when the batch
of bacitracin is released and no refrigeration is required. Partially puri-
fied concentrates (neutral or slightly acid) of bacitracin were stable for 8
to 12 months when stored at 0 to 5°C., according to Anker and his
coworkers.[1] However, there was a 30 to 50 per cent loss in activity fol-
lowing storage at room temperature for 2 weeks. Bacitracin was stable
in 0.01 N hydrochloric acid at 0 to 37°C. It was unstable in alkaline solu-
tions, particularly at a pH greater than 9. Crude bacitracin from various
commercial laboratories varied greatly in stability.

Production.—The production of bacitracin is in the developmental
stage at the present time and is carried out by surface culture methods,
using shallow layers of medium.

Organism.—The organism used is the Tracy I strain of *B. subtilis*. It
is a Gram-positive, aerobic, sporeforming organism.

Stock Cultures—Stock cultures should be grown in media such as
tryptone agar or broth, to which no glucose has been added, according to
Anker and his fellow workers,[5] in order to maintain them in the rough or

[1] *Ibid.*
[2] JOHNSON, ANKER, SCUDI, and GOLDBERG, *op. cit.*
[3] JOHNSON, ANKER, and MELENEY, *loc cit*
[4] Food and Drug Administration, Federal Security Agency, Feb. 16, 1948
[5] ANKER, JOHNSON, GOLDBERG, and MELENEY, *loc cit*

mucoid phase and to prevent the formation of the smooth phase that gives rise to small quantities of bacitracin only.

Inoculums —Anker and his associates[1] reported that cultures grown for 3 days on the surface of tryptone agar, or in 1 per cent tryptone broth, or spore suspensions were satisfactory for use in inoculating the production media.

Media.—Various nonsynthetic media have been used for the production of bacitracin, among them tryptone, beef infusion, savita,[1] amigen, and soybean digest broths. Corn-steep liquor or 1 per cent glucose added to the tryptone or amigen media did not increase the yields of bacitracin.

A synthetic medium of the following composition (concentration in 1 liter of distilled water) was also used by Anker and his fellow workers:

l-Glutamic acid	5 0 g
KH₂PO₄	0 5 g
K₂HPO₄	0 3 g
MgSO₄ 7H₂O	0 2 g
MnSO₄ 4H₂O	0 01 g
NaCl	0 01 g
FeSO₄ 7H₂O	0 01 g
CuSO₄ 5H₂O	0 01 g
Ca(H₂PO₄)₂	2 ml sat sol in dist H₂O

The pH of the medium was adjusted to 6 8 to 7 0 with sodium hydroxide. The medium was then distributed in 200-ml quantities into 1-liter Blake bottles and autoclaved at 15 lb steam pressure for 20 min Sufficient concentrated glucose solution, sterilized by passage through a Chamberland or Seitz filter, was then added aseptically to each bottle to give a concentration of 1 per cent

Anker and his associates[2] endeavored to find substitutes for *l*-glutamic acid and glucose   *d*-Glutamic acid, asparagine, and glycine were unsatisfactory for various reasons   Fructose, sucrose, and lactose could be used in place of glucose, but possessed no special advantages from the yield viewpoint

Conditions of Fermentation.—The antibiotic may be produced at room temperature or 37°C , however, the rate of formation is more rapid at the latter temperature   Maximum titers (yields) of the antibiotic are obtained after 3 to 5 days at 37°C   Anker et al[2] reported that incubation for periods longer than 96 hr   sometimes resulted in autolysis of the pellicle with a subsequent decrease in titer, which may be as high as 50 per cent in 24 hr

JOHNSON, ANKER, & CO., and GOLDBERG, *op cit.*
ANKER, JOHNSON, GOLDBERG, and MELENEY, *loc. cit.*
Ibid

Harvesting.—Although the pH of the medium at the time of harvest depends upon its composition and other factors and is thus different for different media, there is a relation between the final pH and the titer.[1] The pH of the tryptone broth (also the soybean digest) at the time of maximum titer may be 7.8 to 8; that of the synthetic medium, 7.0 to 7.2.

The liquid portion of the fermentation medium is separated from the pellicle by decantation and clarified by filtration through "standard supercel."

Concentration.—The clarified fermentation medium is extracted twice with n-butanol, using one-half volume of the solvent for each extraction. The butanol extract is separated by decantation, filtered, and vacuum-distilled in the presence of water (500 ml. for each 1,000 ml. of butanol extract when the distillation temperature is 28 to 30°C.). Concentration is continued until the butanol has been completely distilled and the volume of the aqueous phase is approximately 1 per cent of that of the original volume of the fermentation liquor. It is important to distill the extract in a sufficient quantity of water, for anhydrous butanol rapidly inactivates bacitracin.

Purification.—Anker and associates[1] used the following method for purifying bacitracin in the laboratory: One volume of a 50 per cent butanol and 50 per cent peroxide-free ether mixture is added to 1 volume of the aqueous concentration and the mixture is agitated in a separatory funnel. Concentrated hydrochloric acid is then added dropwise until the mixture separates into two layers immediately after shaking. The upper solvent layer extracts impurities and becomes discolored; the lower aqueous layer contains the antibiotic. The pH of the lower layer should lie between 3 and 4. It is separated out by decantation and the extraction is repeated. After standing for 10 min., the pH of the aqueous layer is ascertained (it should lie between 3 and 4). The aqueous layer is again decanted and then extracted five times with peroxide-free ether. The butanol and ether are removed from the aqueous layer by vacuum distillation. The pH of the aqueous concentration is adjusted to 6 to 7 with sodium bicarbonate. It may then be dried by lyophilizing.

In another method, magnesium oxide is used to precipitate carbohydrates and other impurities, and the antibiotic is precipitated with salicylic acid.

Unit of Activity ... ...ivalent to 0.026 mg.
(26 micrograms) of rd,
according to the Food and Drug Administration Tentative ... ...ns for Bacitracin.[2]

[1] Ibid.

[2] Dated Feb. 16, 1948.

Assay Methods.—A number of methods for assaying the potency of bacitracin have been reported. A serial-dilution method using the Chanin strain of Group A hemolytic streptococcus has been described by Anker and associates,[1] Details of a plate assay in which *Micrococcus flavus* is employed as the test organism and of a turbidimetric assay in which *Staph. aureus* (PCI 1203) is used as the test organism are described in the Food and Drug Administration Tentative Specifications.[2]

Yields.—Highest yields were obtained from the soybean digest medium and the synthetic medium containing *l*-glutamic acid, average titers of 12 and 6 units per ml, respectively, being obtained Average yields from the tryptone, beef infusion, amigen, and savita media, were 2 units per ml in each case Occasionally yields from these media were two or more times greater

Uses.—Reports concerning the therapeutic uses of bacitracin have been made by Meleney and Johnson;[3] Meleney,[4] Miller, Slatkin, and Johnson;[5] and others.

Meleney and Johnson stated that favorable responses were observed in 88 out of 100 cases of surgical infections treated with bacitracin They examined aerobic and anaerobic cocci isolated from the infections and found that most of them were susceptible to bacitracin and penicillin However, 30 of the organisms were resistant to penicillin and susceptible to bacitracin; six were resistant to bacitracin and susceptible to penicillin, and a few were resistant to both penicillin and bacitracin They also reported that bacitracin was not irritating or toxic locally, and that it was not inhibited by blood, plasma, pus, broken-down tissue, or bacteria that produce penicillinase

Meleney described the successful use of bacitracin in two cases of infections resistant to penicillin and in one case where the patient was allergic to sulphonamides and penicillin

Miller and associates described the local use of bacitracin and found that an ointment of bacitracin containing 480 units per g was effective against superficial pyogenic infections They found some evidence of the development of strains resistant to bacitracin, but no indication of bacitracinase production

AUREOMYCIN

Aureomycin was discovered by Duggar,[6] Lederle Laboratories Division, American Cyanamid Company Preliminary studies indicate that

ANKER, JOHNSON, GOLDBERG, and MELENEY, *loc. cit.*
[1] Dated Feb 16, 1948
[2] MELENEY and JOHNSON, *loc. cit.*
[3] MELENEY, F. L., Report to Surgeon General, July 1, 1947
[4] MILLER, SLATKIN, and JOHNSON *loc. cit.*
[5] ANON., New Patent Antibiotic, *Science News Letter* July 31, 1948

it is effective in combating microorganisms and diseases against which penicillin and streptomycin are not effective. Particular promise has been shown in respect to its use in combating bacterial eye infections; Q fever and Rocky Mountain Spotted Fever (rickettsial diseases); and lymphogranuloma venereum, a venereal disease caused by a virus.

Some References Concerning the Antibiotics

PENICILLIN

ABRAHAM, E P , E. CHAIN, C M FLETCHER, A. D. GARDNER, N. G. HEATLEY, M A JENNINGS, and H. W. FLOREY: Further Observations on Penicillin, *Lancet*, 2: 177–188, 189 (1941)

ANON. "The Story of Penicillin," 29 pp., Merck and Company, Inc , Rahway, N J

ANON : Antibiotics, *Research Today*, 2 (No. 2): 19–30 (1945)

ANON.: Biochemistry of Penicillin and Other Antibiotics; *Research Today*, 2 (No 2) 31–36 (1945)

ANON . "Penicillin: Its Properties, Uses, and Preparation," The Pharmaceutical Press, London. 1946

ANON.: Administration and Dosage of Penicillin, *The Bristol Digest* (Special Issue), 3 (No. 5): 1–20 (1947).

BACKUS, M P., J. F. STAUFFER, and M. J. JOHNSON: Penicillin Yields from New Mold Strains, *Jour. Am. Chem. Soc* , 68: 152–153 (1946)

BARNES, R. B., R. C. GORE, E. F WILLIAMS, S. G. LINSLEY, and E. PETERSON: "Infrared Analysis of Penicillins," Paper presented at Conference on Antibiotic Research held at Washington, D. C., on Jan 31 and Feb. 1, 1947, under the auspices of the Antibiotics Study Section, National Institute of Health.

BENEDICT, R. G., and A. F LANGLYKKE: Antibiotics, *Ann Rev Microbiol* , 1: 193–236 (1947).

BERGER, F. M.: Extraction and Purification of Penicillin, *Nature*, 154: 459 (1944)

BOWDEN, J. P., and W. H PETERSON: The Role of Corn Steep Liquor in Production of Penicillin, *Arch Biochem* , 9 (No. 3): 387–399 (1946)

BRUNEL, J.: Qui a découvert la pénicilline?, *Rev. can biol.*, 3: 333–343 (1944)

CALLAHAN, J. R.: Penicillin Large-scale Production by Deep Fermentation, *Chem Met. Eng* , 51 (No. 4). 94–98 (1944).

———: Penicillin by Deep Fermentation, *Chem Met. Eng* , 51 (No. 4): 130–133 (1944).

CATCH, J R , A. H. COOK, and I M HEILBRON: Purification and Chemistry of Penicillin, *Nature*, 150: 633–634 (1942).

CHAIN, E , and H W FLOREY: Penicillin, *Endeavour*, 3 (9) (1944).

CHAIN, E., H W. FLOREY, A D GARDNER, N G HEATLEY, M. A JENNINGS, J. ORR-EWING, and A. G. SANDERS: Penicillin as a Chemotherapeutic Agent, *Lancet*, 2: 226–228 (1940).

CHALLINOR, S. W : Production of Penicillin, *Nature*, 150: 688 (1942)

——— and J. MACNAUGHTON: The Production of Penicillin, *Jour. Path Bact* , 55: 441–446 (1943).

CLUTTERBUCK, P W., R. LOVELL, and H RAISTRICK: Studies in the Biochemistry of Micro-organisms. XXVI. The Formation from Glucose by Members of the *Penicillium chrysogenum* Series of a Pigment, an Alkali-soluble Protein and Penicillin—The Antibacterial Substance of Fleming, *Biochem Jour.*, 26: 1907–1918 (1932).

COGHILL, R. D.: Penicillin Science's Cinderella, *Chem. Eng. News*, 22 (No. 8): 588–593 (1944).

———, A. E OSTERBERG, and G R HAZEL: The Relative Effectiveness of Pure Penicillins G, X, and K, *Science*, 103: 709–710 (1946).

Committee on Medical Research, O S R D, and the Medical Research Council, London: Chemistry of Penicillin, *Science* 102 (No 2660): 627–629 (1945).

COOK, R.P., W.J. TULLOCH, M.B BROWN, and J BRODIE The Production of Penicillin on Media Made from Vegetable Extracts, Particularly Extracts of Peas, *Jour Path. Bact*, 54: 555 (1944).

DA ROCHA, R V.: Contribuicão ao Estudo da Penicilina, Tese Fac. de Med. de Porto Alegre, Rio Grande do Sul, (114 pp) Brazil, 1943.

DOLKART, R. E., P. L. DEY, and G X SCHWEMLEIN. Penicillin Assay Techniques, A Comparative Study, *Jour Bact*, 53: 17–24 (1947)

DONOVICK, R., D LAPEDES, and F PANSY Studies on the Quantitative Differential Analysis of Mixtures of Several Essentially Pure Penicillin Types, *Jour. Bact*, 54 (No 4) 425–442 (1947)

DUFRENOY, J, and R. PRATT Cytochemical Mechanisms of Penicillin Action. I Oxidation-Reduction Levels, *Jour Bact*, 53: 637–666 (1947)

DUVIGNEAUD, V, F H CARPENTER, R W HOLLEY, A H LIVERMORE, and J R RACHELE: Synthetic Penicillin, *Science*, 104 (No 2706): 431–433, 450 (1946)

EAGLE, H . The Relative Activity of Penicillins F, G, K, and X Against Spirochetes and Streptococci in Vitro, *Jour Bact* , 62: 81–88 (1946)

——— and A MUSSELMAN The Low Therapeutic Activity of Penicillin K Relative to that of Penicillins F, G, and X, and Its Pharmacological Basis, *Science*, 103: 618–620 (1946)

EISSER, H, and B PORZECANSKI Inactivation of Penicillin by Zinc Salts, *Science*, 103 (No 2681) 629–630 (1946)

ELDER, A L. The Commercial Production of Penicillin, *Chem Ind*, 54: 501–504 (1944)

——— Penicillin, *Scient Monthly*, 58: 405–409 (1944)

ENGLIS, W H The Industrial Development of Antibiotics, *Chem Eng News*, 26 (No 18): 1264–1267 (1948)

FLEMING, A On the Antibacterial Action of Cultures of a Penicillium, with Special Reference to Their Use in the Isolation of B Influenzae, *Brit Jour Path*, 10: 226–236 (1929)

On the Specific Antibacterial Properties of Penicillin and Potassium Tellurite, *Jour Path Bact* 35: 831–842 (1932)

Mode of Action of Penicillin, *Nature*, 145: 757 (1940)

In Vitro Tests of Penicillin Potency, *Lancet*, 1: 732–733 (1942)

The Discovery of Penicillin, *Brit Med Bull*, 2 (No 4) 4–5 (1944)

Penicillin Its Practical Application, The Blakiston Company, Philadelphia, 1946

FLOREY, H E, and H W FLOREY General and Local Administration of Penicillin, *Lancet* 1: 387–397 (1943)

FLOREY, H W Penicillin Its Development for Medical Uses, *Nature* 183: 40–42 (1944)

——— and M A JENNINGS Some Biological Properties of Highly Purified Penicillin, *Brit Jour Expt Path*. 23: 120–123 (1942)

FOSTER, J W Quantitative Estimation of Penicillin *Jour Biol Chem* 144: 285–286 (1942)

———: Microbiological Aspects of Penicillin. I. Methods of Assay, *Jour. Bact*, 46: 187–202 (1913).

———: Microbiological Aspects of Penicillin. IV. Procedure for the Cup Assay of Penicillin, *Jour. Bact.*, 47: 43–58 (1913).

———, and E O. Kanow: Microbiological Aspects of Penicillin VIII. Penicillin from Different Fungi, *Jour. Bact.*, 49 (No. 1): 19–29 (1945).

———, H. B Woodruff, and L E. McDaniel: Microbiological Aspects of Penicillin III. Production of Penicillin in Surface Cultures of *Penicillium notatum, Jour Bact*, 46: 421–433 (1913).

———, ———, and ———: Microbiological Aspects of Penicillin. IV. Production of Penicillin in Submerged Cultures of *Penicillin notatum, Jour. Bact.*, 51: 465–478 (1916).

———, ———, D. Perlman, L. E. McDaniel, B. L. Wilker, and D. Hendlin: Microbiological Aspects of Penicillin, IX. Cottonseed Meal as a Substitute for Corn Steep Liquor in Penicillin Production, *Jour. Bact*, 51: 695–698 (1946).

Gailey, F B, J. J. Stefaniak, B. H. Olson, and M. J. Johnson: A Comparison of Penicillin-producing Strains of *Penicillium notatum-chrysogenum, Jour. Bact*, 52: 129–140 (1946).

Goodall, R. R, and A A Levi: A Microchromatographic Method for the Detection and Approximate Determination of the Different Penicillins in a Mixture, *Nature*, 158: 675–678 (1946)

——— and ———: A Micro-chromatographic Method for the Detection and Approximate Determination of the Different Penicillins in a Mixture, *Analyst*, 72: 277–288 (1917).

Hansen, H N, and W. C Snyder: Relation and Dual Phenomenon in *Penicillium notatum* to Penicillin Production, *Science*, 99: 264–265 (1944).

———: Observations on the Mechanism of Action of Penicillin. *Science*, 80: 281–285 (1942)

Herrell, W E: "Penicillin and Other Antibiotic Agents," W. B Saunders Company, Philadelphia, 1946

Higuchi, K, and W H. Peterson: Estimation of Types of Penicillin in Broths and Finished Products A Microbiological Method, *Anal. Chem*, 19 (No. 1): 68–71 (1947)

———, F. G Jarvis, W H. Peterson, and M A Johnson: Effect of Phenylacetic Acid Derivatives on the Types of Penicillin Produced by *Penicillium chrysogenum* Q176, *Jour. Am. Chem. Soc* 68: 1669–1670 (1946)

Hobby, G I, T F Lenert, and B Hyman: The Effect of Impurities on the Chemotherapeutic Action of Crystalline Penicillin, *Jour Bact*, 54 (No 3): 305–323 (1947)

Hunter, A C, and W A. Randall: Standardization of Assay of Penicillin, *Jour Assoc. Offic Agr Chemists*, 27 (No. 3): 430–438 (1944).

Johnson, M J.: Metabolism of Penicillin-Producing Molds, *Ann. N.Y Acad. Sci*, 48 (Art. 2). 57–66 (1946)

Joslyn, D. A, Penicillin Assay. An Outline of Four Hour Turbidimetric Method, *Science*, 99: 21. 1944.

Kavanagh, F : A Qualitative Test for Penicillin, *Science*, 105: 189 (1947).

———: Antibacterial Substances from Fungi and Green Plants, *Advances in Enzymol*, 7: 461–511 (1947)

Keefer, C.S., lin in the   Council: Penicil-
                                                                                              17 (1943)

KNIGHT, S. G., and W. C. FRAZIER The Control of Contaminants in Penicillin Fermentations by Antiseptic Chemicals, *Jour Bact*, 50, 505-516: 1945

KOCHOLATY, W, Cultural Characteristics of *Penicillium notatum* in Relation to the Production of Antibacterial Substances: Induction of the Dual Nature of the Antibacterial Substance, *Jour Bact*, 44: 469-477 1942

KOFFLER, H. E, S G KNIGHT, and W. C FRAZIER. The Effect of Certain Mineral Elements on the Production of Penicillin in Shake Flasks, *Jour Bact*, 52 (No 1). 115-123 (1947).

——, R. L EMERSON, D PERLMAN, and R H BURRIS Chemical Changes in Submerged Penicillin Fermentations, *Jour Bact*, 50: 517-548 (1945)

——, S. G KNIGHT, R L EMERSON, and R H BURRIS The Effect of Certain Chemicals on Penicillin Production and Mold Metabolism in Shake Flask Fermentations, *Jour Bact*, 50: 549-559 (1945)

KOLMER, J A "Penicillin Therapy, Including Streptomycin, Tyrothricin and Other Antibiotic Therapy," 2d ed , D Appleton-Century Co , Inc , New York, 1947

LEDERLE; "Excerpts and Conclusions from the Literature on the Principal Uses for Penicillin," Lederle Laboratories Division, American Cyanamid Co , New York, 1948

LEE, S W , E J FOLEY, J A EPSTEIN, J H WALLACE, JR Improvements in the Turbidimetric Assay for Penicillin, *Jour Biol Chem*, 152: 485-496 (1944)

LIBBY, R L, and N L HOLMBERG The Activity of Penicillins G and X In Vitro, *Science*, 102: 303-304 (1945)

McMAHAN, J R An Improved Short Time Turbidimetric Assay for Penicillin, *Jour Biol Chem*, 153: 249-258 1944

MOORE, J E "Penicillin in Syphilis," Charles C Thomas, Publisher, Springfield, Ill , 1946

MOYER, A J, and R D COGHILL Penicillin VIII Production of Penicillin in Surface Culture, *Jour Bact*, 51 (No 1) 57-78 1946

—— and ——, Penicillin IX The Laboratory Scale Production of Penicillin in Submerged Culture by *Penicillium notatum* Westling (NRRL 832), *Jour Bact*, 51 (No 1) 79-93 1946

—— and ——, Penicillin X The Effect of Phenylacetic Acid on Penicillin Production, *Jour Bact*, 53 (No 3) 329-341 (1947)

NAEFE, R G "Antibiotic Substances Their Biological and Chemical Properties" 2d ed , Antibiotics Study Section Division of Research Grants and Fellowships, National Institute of Health Bethesda, Md , February 1948

National Academy of Sciences "The Chemistry of Penicillin, Princeton University Press, Princeton, N J 1948

ORY, E M, M MEADS, and M FINLAND Penicillin X, *Jour Am Med Assoc* 129: 257-261 (1945)

PRATT, R Influence of Phosphate on Stability of Crude Penicillin *Plant Physiol* 22 (No 3) 308-314 (1947)

—— Influence of Phosphate on Stability of Partially Purified Penicillins *Jour Am Pharm Assoc Sci Ed* 36 No 3 64-72 1947

—— and J DUFRENOY Practical Three-hour and Two-hour Cylinder Plate Assays for Penicillin, *Nature*, 159: 576 1947

—— and —— Cytochemical Interpretation of the Mechanism of Penicillin Action *Bact Rev*, 12 (No 1 79-103 1948

——, and P A SMITH The Enhancement of Penicillin Effectiveness in Minute Traces of Cobalt, *Jour Bact* 55 No 1 75-77 1948

PEARL, I. A , and J. W. APPLING: Penicillin Production, *Science* (N S.), **100**: 51 (1944)

RAKE, G., and A. P. RICHARDSON: Pharmacology of Penicillin, *Ann. N.Y. Acad Sci*, **46** (Art. 2): 143–174 (1946).

——, W. B DUNHAM, and R. DONOVICK: The Evaluation of Antibiotics by the Prevention of Experimental Syphilis, *Jour. Infectious Diseases*, **81**: 122–129 (1947)

RAMMELKAMP, C. H.: A Method for Determining the Concentration of Penicillin in Body Fluids and Exudates, *Proc. Soc. Expt. Biol. Med*, **51**: 95–97 (1942)

RAPER, K. B.: The Development of Improved Penicillin-producing Molds, *Ann N Y Acad. Sci*, **48** (Art. 21: 41–56 (1946).

—— and D. F. ALEXANDER: The Preservation of Molds by the Lyophil Process, *Mycologia*, **37** (July–August, 1945).

—— and ——: Penicillin: V. Mycological Aspects of Penicillin Production, *Jour. Elisha Mitchell Sci. Soc*, **61**: 74–113 (1945).

——, and R. D. COGHILL: Penicillin: II. Natural Variation and Penicillin Production in *Penicillium notatum* and Allied Species, *Jour. Bact*, **48** (No 6): 639–659 (1944).

—— and R D. COGHILL: "Home-made" Penicillin, *Jour. Am. Med. Assoc*, **123**: 1135 (1945).

—— and D. I FENNELL: The Production of Penicillin X in Submerged Culture, *Jour. Bact.*, **51**: 761–777 (1946).

REID, D : A Study of the Bactericidal, Bacteriolytic or Inhibitory Substance Produced by Some Molds and Some Factors Which Influence Its Production, *Jour. Bact*, **25**: 31 (1933)

——: The Ability of a Mold or Its Metabolic Products to Inhibit Bacterial Growth, *Jour. Bact*, **27**: 28 (1931).

——. Some Properties of n Bacterial Substance Produced by a Mold, *Jour. Bact*, **29**: 215–221 (1935)

REEVES, M. D , and W H SCHMIDT: Penicillin. IV. A Device for Placing Cylinders on Assay Plates, *Jour Bact*, **49** (No. 4): 395–400 (1945).

ROBERTS, E. C , C K CAIN, R D MUIR, F J REITHEL, W. L GOBY, J T VAN BRUGGEN, D M HOMAN, P. A KATZMAN, L R JONES, and E. A. DOISY: Penicillin B, An Antibacterial Substance from *Penicillium notatum*, *Jour. Biol Chem*, **147**: 47–58 (1943).

SALIVAR, C. J , V V BOGERT, and E V BROWN. Separation and Identification of Penicillin Species in Commercial Penicillin (Q-176 Culture), Paper presented at Conference on Antibiotic Research, Washington, D. C , Jan. 31, and Feb. 1, 1947, under auspices of the Antibiotics Study Section, National Institute of Health

SAVAGE, G M , and M J VANDER BROOK. The Fragmentation of the Mycelium of *Penicillium notatum* and *Penicillium chrysogenum* by a High Speed Blender and the Evaluation of Blended Seed, *Jour. Bact*, **52**: 385–391 (1946)

SCHMIDT, W. H , and A J. MOYER: Penicillin I Methods of Assay, *Jour. Bact*, **47** (No 2): 199–210 (1944).

——,   Effect of Dissociation Phase
——   of   **49**: 411–412 (1945)
SHEEHA   ical Assay Method for Penicill
lin

SMITH, E. C : *Jour Soc Chem Ind* , **65**: 303–314 (1946).

SMITH, L : The Bacteriostatic Agent of *Penicillium chrysogenum*, *Franklin Inst. Jour* , **234**: 396–402 (1942).

SOLLMANN, T : "A Manual of Pharmacology and Its Applications to Therapeutics and Toxicology," 7th Ed , W B Saunders Company, Philadelphia, 1948

SRINIVASA, R. S.: Production of Penicillin, *Nature*, 154: 83 (1944).

STEFANIAK, J. J., F B GAILEY, C S BROWN, and M J JOHNSON: Pilot Plant Equipment for Submerged Production of Penicillin, *Ind Eng Chem*, 38: 666–671 (1946).

———, ———, F G JARVIS, and M J JOHNSON. The Effect of Environmental Conditions on Penicillin Fermentations with *Penicillium chrysogenum* X-1612, *Jour Bact*, 52: 119–127 (1946)

STONE, R W., and M A FARRELL. Synthetic Media for Penicillin Production, *Science*, 104 (No 2706): 445–446 (1946)

STRAIT, L A J, DUFRENOY, R PRATT, and V LAMB Enhancement of Penicillin Effectiveness by Traces of Cobalt. *Jour Am Pharm Assoc, Sci Ed*, 37: 133–135 (1948)

TAYLOR, H G : Growth of *Penicillium notatum* on Various Media and the Development of An Antibacterial Substance. *Proc Soc Expt Biol Med*, 52: 299–301 (1943)

TAYLOR, T H M · Penicillin, *Chem Eng Prog, Trans Sect*, 43 (No 4): 155–159 (1947).

The Editorial Board of the Monograph on the Chemistry of Penicillin The Chemical Study of Penicillin A Brief History, *Science*, 105 (No. 2739): 653–659 (1947)

THOM, C, and K B RAPER "A Manual of the Aspergilli," The Williams & Wilkins Company, Baltimore, 1945

TOURETTE, R, S SHULTZ, and W McDERMOTT The Relations of Protein Binding to the Pharmacological and Antibacterial Activity of Penicillins X, G, Dihydro F, and K, *Jour Bact*, 53: 581–595 (1947)

VAN WINKLE, W, and R P HERWICK Penicillin—A Review, *Jour Am Pharm Assoc, Sci Ed*, 34: 97–109 (1945)

VERDUYN, W F, and A K MILLER The Effect of Caronamide upon Penicillin Therapy of Experimental Pneumococcus and Typhoid Infections in Mice, Paper presented at Conference on Antibiotic Research, Washington, D C, Jan 31, and Feb. 1, 1947, under the auspices of Antibiotics Study Section, National Institute of Health

——— and ——— The Effect of Caronamide upon Penicillin Therapy of Experimental Pneumococcus and Typhoid Infections in Mice, *Jour Bact*, 53: 670 (1947)

WAKSMAN, S A, and E HORNING Distribution of Antagonistic Fungi in Nature and Their Antibiotic Activity *Mycologia*, 35: 47–65 (1943)

——— and H C REILLY Strain Specificity and Production of Antibiotic Substances III *Penicillium notatum–chrysogenum* group, *Proc Nat Acad Sci*, 30: 99–105 (1944)

WELCH, H, R H HERWICK, and A C BUNTER Penicillin Properties and Standardization, *Jour Am Pharm Assoc, Pract Pharm Ed*, 6 (No 4): 93–96 (1945)

—, W A RANDALL, and E MARTIN Methods of Testing Antibiotic Substances and Limitations Involved *Jour Am Pharm Assoc, Sci Ed*, 35 No 4: 102–113 1946

—, E E PUTNAM W A RANDALL, and R P HERWICK Penicillin X Successful Treatment of Gonorrhea in a Single Intramuscular Injection, *Jour Am Med Assoc*, 126: 1024 1944

WINNER, H A Penicillin and Streptomycin A Review of Their Current Uses, *Jour Kansas Med Soc*, June 1947

WHIFFEN, A J, and G M SAVAGE The Relation of Natural Variation in *Penicillium notatum* to the Yield of Penicillin in Surface Culture *Jour Bact*, 53: 231–240 (1947)

WHITE, A. G. C., L. O. KRAMPITZ, and C. H. WERKMAN: On a Synthetic Medium for the Production of Penicillin, *Arch. Biochem.*, 8: 303–311 (1945).

WOODRUFF, H. B., and J. W. FOSTER: Microbiological Aspects of Penicillin. VII. Bacterial Penicillinase, *Jour. Bact.*, 49: 7–17 (1944).

STREPTOMYCIN

ALEXANDER, H. E., G. LEIDY, G. RAKE and R. DONOVICK: Hemophilus Influenzae Meningitis Treated with Streptomycin, *Jour. Am. Med. Assoc*, 132: 434–440 (1946).

BENEDICT, R. G., and A. F. LANGLYKKE: Antibiotics. *Ann. Rev. Microbiol*, 1: 193–236 (1947).

BENNETT, R. E.: Nutrition of *Streptomyces griseus* in Relation to Streptomycin Titer, *Jour. Bact.*, 53: 254 (1947).

BONDI, A., JR., C. C. DIETZ, and E. H. SPAULDING: Interference with the Antibacterial Action of Streptomycin by Reducing Agents, *Science*, 103: 399–401 (1946).

BOXER, G. E., V. C. JELINEK, and P. M. LEGHORN: The Colorimetric Determination of Streptomycin in Clinical Preparations, Urine and Broth, *Jour. Biol. Chem*, 169: 153–165 (1947).

BRINK, N. G., F. A. KUEHL, JR., and K. FOLKERS: Streptomyces Antibiotics III. Degradation of Streptomycin to Streptobiosamine Derivatives, *Science*, 102: 506–507 (1945).

———, ———, E. H. FLYNN, and K. FOLKERS: Streptomyces Antibiotics XI. The Structure of Tetraacetylbisdesoxystreptobiosamine, *Jour. Am. Chem. Soc*, 68: 2405 (1946).

———, ———, ———, and K. FOLKERS: Streptomyces Antibiotics. XII. The Degradation of Streptomycin and Dihydrostreptomycin with Methanol, *Jour. Am. Chem. Soc*, 68: 2557 (1946).

CARTER, H. E., R. K. CLARK, JR., S. R. DICKMAN, Y. H. LOO, P. S. SKELL, and W. A. STRONG. Isolation and Purification of Streptomycin, *Jour. Biol. Chem*, 160: 337–342 (1945).

CARVAJAL, F. Studies on the Structure of *Streptomyces griseus*, *Mycologia*, 38: 587–595 (1946).

Committee on Chemotherapeutics and Other Agents, National Research Council, C. S. Keefer, Chairman. Streptomycin in the Treatment of Infections, *Jour. Am. Med. Assoc*, 132: 4–11, 70–77 (1946).

DENKEWALTER, R., M. A. COOK, and M. TISHLER: The Effect of Cysteine on Streptomycin and Streptothricin, *Science*, 102: 12 (1945).

DONOVICK, R., D . . . . Method of
    Assaying Stre I
——— and G. RA. 16
    Modifications . . . . ur.
——— and ——— .
    Bact., 53 (No. 2). 205–211 (1947)

———, ———, and J. FRIED: The Influence of Certain Substances on the Antibiotic Activity of Streptomycin In Vitro. II. The Action of Some Carbonyl Reagents on Streptomycin, *Jour. Biol. Chem.*, 164 (No. 1): 173–181 (1946).

ENGELS, W. H.: The Industrial Production of Streptomycin, Paper presented at A.A.A.S. Symposium on Antibiotics, Chicago, Dec. 29–30, 1947.

———: The Industrial Development of Antibiotics, *Chem. Eng. News*, 26 (No. 18): 1284–1287 (1948).

Food and Drug Administration, Federal Security Administration: Title 21, Food and Drugs, Chap. I, Parts 141 and 146, Test and Methods of Assay of Antibiotic Drugs, Penicillin and Streptomycin and Certification of Batches of Penicillin and Streptomycin-containing Drugs, *Federal Register*, Apr 4, 1947

FRIED, J., and G. A. BOYACK, and O WINTERSTEINER Streptomycin The Chemical Nature of Streptidine, *Jour. Biol Chem*, 162 (No 2, 391-392 (1946)

—— and H. E. STAVELY: Streptomycin V D gradation of Streptomycin B to Streptidine, Streptobiosamine and D-Mannose, *Jour Am Chem Soc*, 69: 1549 (1947).

—— and E. Titus: Streptomycin B, An Antibiotically Active Constituent of Streptomycin Concentrates, *Jour Am Chem Soc*, 168 (No 1 391-392 (1947).

——, D E. WALZ, and O WINTERSTEINER Streptomycin III 4-Desoxy-L-Erythrose (Threose) Phenylosazone from Streptobiosamine *Jour Am. Chem Soc*, 68: 2746 (1946)

—— and O WINTERSTEINER The Composition of Streptomycin Reineckate, *Science*, 104: 273-274 (1946)

—— and ——— Streptomycin II Reduction and Oxidation Products of Streptomycin and of Streptobiosamine. *Jour Am Chem Soc*, 69: 79-86 (1947)

GEIGER, W B., S R GREEN, and S A WAKSMAN The Inactivation of Streptomycin and Its Practical Applications. *Proc Soc Expt Biol Med*, 61, 187-192 (1946)

GORDON, A M. Streptomycin in Tularemia, *Jour Am Med Assoc*, 132: 21-22 (1946).

GOTTLIEB, D., and H W ANDERSON Morphological and Physiological Factors in Streptomycin Production, *Bull Torrey Botan Club*, 74 (No 4, 293-302 (1947)

GYÖRGY, P., and H F LEY Chemotherapy Penicillin, Sulfonamides, Streptomycin, and Tyrothricin, *Advances in Pediatrics* 2: 151 193 (1947)

HARRIS, D., G RAKE, and R DONOVICK Bactericidal Action of Streptomycin, *Proc Soc Expt Biol Med*, 62: 25 31 (1946)

HEILMAN, D H., A Method for Estimating the Concentrations of Streptomycin in Body Fluids, *Proc Staff Meetings Mayo Clinic*, 20: 145 150 (May 16, 1945)

HEILMAN, F R Streptomycin in the Treatment of Experimental Infections with Micro-organisms of the Friedländer Group (Klebsiella), *Proc Staff Meetings Mayo Clinic*, 20: 32 39 (Feb 7, 1945)

HERRELL, Wallace E "Penicillin and Other Antibiotic Agents," W B Saunders Company, Philadelphia and London, 1946 Special reference is made to Chaps XXIII and XXVI

HINSHAW, J W, and C W BINES The Present Status of Streptomycin, *N I State Jour Med*, 47 (No 11: 1276-1284 (1947)

HOBBY, G L , and T F LANERT Observations on the Action of Streptomycin in vitro (II), *Proc Soc Expt Biol Med*, 65. 212 219 (1947)

JONES, D., H J METZLER, A SCHATZ and S A WAKSMAN Control of Gram-Negative Bacteria in Experimental Animals by Streptomycin *Science* 100: 103 104 (1944)

KIRKPATRICK, S D A Case Study in Biochemical Engineering, *Chem Eng*, 54 (No 11: 91 96 (1947)

——— The Story of Streptomycin, *Chem Eng* 54 (No 11: 97 102 (1947)

KUEHL, F A Jr, E H FLYNN N G BRINK, and K FOLKERS Streptomyces Antibiotics X The Degradation of Streptomycin and Dihydrostreptomycin with Ethyl Mercaptan *Jour Am Chem Soc* 68: 2096 2101 (1946)

——, ——— and K FOLKERS Streptomyces Antibiotics XIII The Structure of Streptobiosamine, *Jour Am Chem Soc*, 68: 2679 2684 (1946)

———, ———, F. W. Holly, R. Mozingo, and K Folkers: Streptomyces Antibiotics. V. N-Methyl-l-Glucosamine from Streptomycin, *Jour. Am. Chem. Soc*, 68. 536 (1916).

———, R L. Peck, C. E. Hoffhine, Jr, R. P. Graber, and K. Folkers: Streptomyces Antibiotics. VII Isolation of Streptomycin, *Jour Am. Chem Soc*, 68: 1460-1462 (1946).

———, ———, ———, E W. Peel, and K. Folkers: Streptomyces Antibiotics XIV. The Position of the Linkage of Streptobiosamine to Streptidine in Streptomycin, *Jour. Am. Chem. Soc*, 69: 1234 (1946).

———, A Walti, and K Folkers: Streptomyces Antibiotics. I. Crystalline Salts of Streptomycin and Streptothricin, *Science*, 102: 34-35 (1945).

Lapedes, D, R. Donovick, and G. Rake: Further Studies on the Testing of Sterility of Concentrated Streptomycin Solutions, *Proc. Soc. Expt. Biol Med*, 64: 269-271 (1947).

Lenert, T. F, and G. L. Honny: Observations on the Action of Streptomycin *in vitro* (I), *Proc Soc Expt. Biol Med*, 65: 235-242 (1947)

LePage, G A, and E. Campbell: Preparation of Streptomycin, *Jour. Biol. Chem*, 162: 163-171 (1945).

Levy, G B., P Schwed, and J. W Sackett: Polarographic Analysis of Streptomycin, *Jour. Am Chem Soc*, 68: 528 (1946).

Loo, Y. H, P S Skell, H H Thornberry, J. Ehrlich, J. M McGuire, G M. Savage, and J. C Sylvester: *Jour. Bact*, 50 (No. 6): 701-709 (1945).

Merck and Company, Inc: "The Story of Streptomycin," 1946, "The Clinical Use of Streptomycin," 1947; "The Effects of Streptomycin in Tuberculosis in Man," 1948, Rahway, N.J.

Molitor, H: Pharmacology of Streptothricin and Streptomycin, *Ann. N.Y. Acad. Sci*, 48 (Art 2): 101-117 (1946).

Murray, R, T. F Paine, and M Finland: Streptomycin. I Bacteriologic and Pharmacologic Effects, *New Eng Jour Med*, 236: 701-712 (May 8, 1947)

Osgood, E E, and S. M Graham: Simple Rapid Method for Assay of Bactericidal and Bacteriostatic Agents, *Am Jour Clin Path*, 17: 93-107 (1947).

Oswald, E J, and J. K Nielsen: Studies on Stability of Streptomycin in Solution, *Science*, 105: 184 (1947)

Paine, T. F, R Murray, and M Finland: Streptomycin II Clinical Uses, *New Eng. Jour. Med*, 236: 748-760 (May 15, 1947)

Parke, Davis & Company: "Streptomycin," Detroit, Mich., 1947

Peck, R L, N. G Brink, F. A Kuehl, Jr, E H F inn, A Walti, and K Folkers. Streptomyces Antibiotics II. Crystalline Streptomycin Trihydrochloride —Calcium Chloride Double Salt, *Jour Am Chem Soc*, 67: 1866 (1945)

———, R P Graber, A Walti, E W Peel, C E Hoffhine, Jr, and K Folkers' Streptomyces Antibiotics. IV Hydrolytic Cleavage of Streptomycin to Streptidine, *Jour. Am Chem. Soc*, 68: 29-31 (1946)

———, C E. Hoffhine, Jr, and K Folkers: Streptomyces Antibiotics IX. Dihydrostreptomycin, *Jour. Am. Chem. Soc*, 68: 1390 (1946)

———, ———, E. W. Peel, R E. Graber, F W Holly, R Mozingo, and K. Folkers: Streptomyces Antibiotics. VII. The Structure of Streptidine, *Jour. Am Chem. Soc*, 68: 776-781 (1946).

Porter, R. W.: Streptomycin Engineered into Commercial Production, *Chem Eng*, 53 (No. 10): 94-98 (1946)

———: Streptomycin Calcium Chloride Complex, *Merck Rept*, July, 1947

Postovskii, I. Ya., and N P Bednyagina: Antibiotics, *Uspekhi Khim*, 16: 3-28 (1917)

Price, C. W., J K Nielsen, and H Welch: The Estimation of Streptomycin in Body Fluids, *Science*, 103: 56-57 (1946)

Priest, W S: The Present Status of Penicillin and the Newer Antibiotics, *Ind Med*, 15: 431-436 (1946).

Rake, G, and R Donovick A Procedure for Testing Sterility of Concentrated Streptomycin Solutions, *Proc Soc Expt Biol Med*, 62: 31-33 (1946)

—— and ——: Studies on the Nutritional Requirements of *Streptomyces griseus* for the Formation of Streptomycin, *Jour Bact*, 52 223-226 (1946)

—— and ——: Features of the Antibacterial Action of Streptomycin, Paper presented at the Meeting of the A.A.A.S., Chicago, Dec 29, 1947

——, D, Hamre, F Kavanagh, W L Koerber, and R Donovick On the Toxicity of Streptomycin, *Am Jour Med Sci*, 210: 61-66 (1945)

——, C M. McKee, F E Pansy, and R Donovick On Some Biological Characteristics of Streptomycin B, *Proc Soc Expt Biol Med*, 65 107 112 (1947)

Rigna, P.P., L A Wassell, and I A Solomons Stability of Streptomycin, *Jour. Biol Chem*, 165: 631-638 (1946)

Robinson, H J, D G Smith, and O E Graessle Chemotherapeutic Properties of Streptomycin, *Proc Soc Expt Biol Med* 57 · 226 231 (1944)

Schatz, A, E Bugie, and S A Waksman Streptomycin, a Substance Exhibiting Antibiotic Activity Against Gram-positive and Gram-negative Bacteria, *Proc Soc Expt Biol Med*, 55: 66-69 (1944)

—— and S A Waksman Effect of Streptomycin and Other Antibiotic Substances upon *Mycobacterium tuberculosis* and Related Organisms, *Proc Soc Expt Biol Med*, 57: 244-248 (1944)

—— and ——— Strain Specificity and Production of Antibiotic Substances IV Variations and Mutations among Actinomyces with Special Reference to *Actinomyces griseus*, *Proc Nat Acad Sci* 31, 129 137 (1945)

Schenck, J R, and M A Spielman Formation of Maltol by Degradation of Streptomycin, *Jour Am Chem Soc*, 67: 2276 (1945)

Scott, L G Streptomycin A Review of the Literature *Delaware State Med Jour*, 18: 18-19 (1946)

Scudi, J V, G E Boxer, and V C Jelinek Color Reaction Given by Streptomycin, *Science*, 104: 486 (1946)

Silcox, H Production of Streptomycin, *Chem Eng News* 24 2763 2764 (1946)

Squibb, E R, and Sons "Streptomycin Therapy," 2d ed , New York 1947

Stanley, A R Improving Streptomycin Yields by Strain Selection and Inoculum Development, *Jour Bact*, 53: 254 (1947)

—— Recent Progress in the Production of Streptomycin and Penicillin Paper presented before Ohio-Michigan Regional A C S meeting in Toledo, Ohio, Mar 15, 1947.

Steinman, H, and H J Robinson A Method for Determination of Streptomycin in Body Fluids, *Proc Soc Expt Biol Med*, 59: 255 257 1945

Titus, E, and J Fried The Use of Counter-current Distribution for the Analysis of Streptomycin Preparations, *Jour Biol Chem*, 168 No 1 393 344 1947

Van Dolah, R W, and G E Christenson Chemical Inactivation of Streptomycin *Arch Biochem*, 12: 7-12 1947

Vischer, W A Lasure, J L Streptomycin, *Med internal (Madrid)*, 9: 448 470 1947

WAKSMAN, S. A.: Production and Activity of Streptomycin, *Jour. Bact*, 46: 299-310 (1943).

———: Standardization of Streptomycin, *Science*, 102 (No 2637): 40-41 (1945).

———: The Discovery and Development of Streptomycin, *Nucleus*, 24 (No 7): 171, 173 (1947).

——— and H. C. REILLY: Agar-streak Method for Assaying Antibiotic Substances, *Ind. Eng Chem*, Anal. Ed., 17: 556-558 (1945).

———, ———, and A. SCHATZ: Strain Specificity and Production of Antibiotic Substances. V Strain Resistance of Bacteria to Antibiotic Substances, Especially to Streptomycin, *Proc. Nat Acad. Sci*, 31: 157-164 (1945).

——— and A SCHATZ: Streptomycin—Origin, Nature and Properties, *Jour. Am Pharm. Assoc*, Sci. Ed., 34 (No. 11): 273-291 (1945).

———, ———, and H. C. REILLY: Metabolism and the Chemical Nature of Streptomyces griseus, *Jour. Bact.*, 51: 753-759 (1946).

——— and H. B WOODRUFF: Selective Antibiotic Action of Various Substances of Microbial Origin, *Jour. Bact*, 44: 373-384 (1942).

WINNEN, H. A.: Penicillin and Streptomycin—A Review of Their Current Uses, *Jour. Kansas Med. Soc*, 48: 261-273 (1947).

BACITRACIN

ANKER, H S, B. A JOHNSON, J. GOLDBERG, and F L. MELENEY: Bacitracin: Methods of Productio
Chemical Pr

EAGLE, H : The '
Man of an Antibiotic Derived from *B. subtilis* (Bacitracin), Abstract of reports presented at Conference on Antibiotic Research held at Washington, D. C., Feb. 1, 1947

Food and Drug Administration, Federal Security Agency: Tentative Specifications for Bacitracin. Standards of Identity, Strength, Quality, and Purity, Washington, D.C., Feb. 16, 1948.

JOHNSON, B. A, H. ANKER, and F. L. MELENEY: Bacitracin: A New Antibiotic produced by a Member of the *B subtilis* Group, *Science*, 102 (No. 2650): 376-377 (1945).

———, ———, J V. SCUDI, and J. GOLDBERG: In Vivo and In Vitro Laboratory Observations on Bacitracin, Mimeograph Report, College of Physicians and Surgeons, Columbia University, New York.

MELENEY, F. L · Copy of the Quarterly Progress Report Sent to Office of the Surgeon General, July 1, 1947.

——— and B. JOHNSON: Bacitracin Therapy. The First Hundred Cases of Surgical Infections Treated Locally with the Antibiotic, *Jour Am Med Assoc*, 133: 675-680 (Mar 8, 1947)

MILLER, J. L, M H. SLATKIN, and B. A JOHNSON. Local Use of Bacitracin, *Jour Investigative Dermatol.*, 10 (No 3). 179-188 (1948)

SCUDI, J. V, and W. ANTOPOL: Some Pharmacological Characteristics of Bacitracin, *Proc Soc Exp Biol Med*, 64: 503 (1947).

———, M. D   Proc Soc.
Bacitracin   '
Expt Biol. Med, 65: 9 (1947).   .

———, I A CORET, and W ANTOPOL: Some Pharmacological Characteristics of Bacitracin III Chronic Toxicity Studies of Commercial Bacitracin in the Dog and Monkey, In press.

TEXTILE MICROBIOLOGY

Textile Fibers.—Textile fibers may be classified as natural and artificial fibers. Natural fibers include plant, animal, and mineral fibers. Among the plant fibers are coir (from the coconut shell), cotton (seed hair); flax, hemp, jute, ramie, and sisal (bast fibers), and other fibers of less commercial importance. Silk and wool are the two most important fibers of animal origin but very different in their mode of origin and in composition. Asbestos fiber (a silicate of magnesium and calcium) is of mineral nature. Rayons (artificial silk) are artificial fibers of vegetable origin. Lanital is an artificial wool made from casein. The production of other new synthetic fibers is very actively pursued at the present time, one being the Nylon which has been developed by the duPont Company.

There are several kinds of rayon: acetate rayon, cuprammonium rayon, and viscose rayon. The latter two rayons are regenerated cellulose.[1] Acetate rayons are less affected by mildew than other rayons.

The fibers of plant origin consist largely of carbohydrate substances, while those of animal origin are proteinous or nitrogenous in nature.

Types of Microorganisms Found on Textile Fibers.—As would be expected, most natural fibers show some microbial association. Bacteria, molds, actinomycetes, and yeasts are consistently found on textile fibers. In the case of plant fibers, these organisms have their origin in soil, water, etc., and as germs ecologically associated with green plants or introduced during processes of separating the fiber from other tissues. The presence of a particular organism may or may not be significant, for most of the normally occurring microorganisms produce no injury under ordinary conditions. Animal fibers also show characteristic types of microbes, some derived from air and soil, others from the bodies of the fiber-producing animals.

Conditions Favoring the Action of Microorganisms.—After the fibers have been separated and commercially baled or packed together changes in moisture content may take place. The availability of moisture and food material, a suitable temperature and pH, and the absence of chemical antiseptics favor the development of fungi and bacteria on commercial fibers and also to some degree on textiles. The fibers are in a sense poten-

tial foods, and as such vary according to wetness or dryness, exposure to air, and other factors.

Types of Destruction Caused by Microorganisms.—Under adverse conditions microorganisms may produce any of the following unfavorable actions: reduction or destruction of the tensile strength of the fibers; discoloration of the fabric owing to the production of pigments, perithecia, spores, or chemical by-products that react adversely with dyes already present; or alteration of the pH of fibers, with the result that the affinity for dyes is changed and the finished fabric may lack luster.

Mildew.—Mildew is a term used to denote the growth of fungi on various substrates.

Tendering.—Tendering, as applied to textiles, implies a weakening of the fibers.

Some Textiles Affected by Microorganisms.—Damage may occur in the raw fibers or in the finished products. Dyed and printed cotton goods, woolen goods, awnings, tarpaulins, tents, fishing nets, and many other articles are subject to the deleterious action of microorganisms unless they are kept dry or treated with chemical agents or otherwise protected.

Literature on the Microbiology of Textiles.—Thaysen and Bunker[1] have reviewed the literature concerning the microorganisms found on cotton and cotton textiles to the year 1927. Much of this literature and many other publications along these lines have appeared in the *Journal of the Textile Institute*. Prindle more recently has carried out extensive research on the microbiology of textile fibers, under the direction of Dr S. C. Prescott. The results of this research, which is concerned principally with cotton and wool microbiology, have been published as a series of articles in *Textile Research*[2] Prindle has reviewed briefly a large number of significant papers.

Excellent discussions of the microbiology of cellulose will be found in Waksman's text on "Soil Microbiology" and in Thaysen and Bunker's text on "The Microbiology of Cellulose, Hemicelluloses, Pectin and Gum." It is obvious that any thorough study of textile microbiology must also involve a study of the microorganisms that attack cellulose.

COTTON

Structure.—The X-ray spectrometer, ultramicroscope, and chemical tests have established much new information in respect to the *structure* and composition of fibers.

[1] Thaysen, A C., and H. J. Bunker, "The Microbiology of Cellulose, Hemicelluloses, Pectin and Gum," Oxford University Press, New York, 1927.
[2] Prindle, B., The Microbiology of Textile Fibres, *Textile Research*, 1933-1936

The mature cotton fiber is hollow and twisted. It is coated with oil and wax, making it impervious to moisture. The individual cotton fiber is made up of fibrils, interlaced chains of elliptical cellulose micelles (approx. 11 by 15 microns) embedded in a colloidal matrix of pectin nature.[1] The physical structure of the cotton fiber has been investigated by Farr,[2] by Farr and Ecker-on,[3] and others.

Approximately 84 per cent of raw cotton is pure cellulose.

Numbers and Kinds of Molds and Bacteria.—Commercial raw cotton is usually highly infected with molds and bacteria.[4] Fresh samples of raw cotton fiber have yielded from 4 to 58 million bacteria and from 120,000 to 400,000 molds per gram.[4]

The molds and bacteria found on raw cotton were largely of the soil type or the type found on fresh plant tissues. Molds found on unstored samples[4] include species of *Hormodendrum*, *Fusarium*, *Alternaria*, *Sporotrichum* and *Mentha*-like organisms, with small numbers of the genera *Aspergillus* and *Penicillium*, bacteria included rod types in large numbers.

Stored samples of raw cotton contained aspergilli and penicillia, and soil types of sporeforming bacteria. Bacteria of the genera *Bacillus* and *Flavobacterium* predominated.[4]

Usually the aspergilli and penicillia isolated were able to utilize cellulose, starch, agar, and gelatin as the only sources of carbon, although rather slowly. The types of molds that predominated on fresh samples of raw cotton grew more readily on cellulose, starch, gelatin, and agar (especially on the first three of these compounds) than the molds found in the stored samples.

According to Prindle, one would expect unstored samples to deteriorate much more rapidly than samples that had been stored.

The bacteria found in raw cotton by Prindle were usually protein digesters which did not demonstrate much action toward carbohydrate media, except for acid formation from glucose media. Hence it would not be expected that they would attack cellulose or starch appreciably.

Bacterial spores present in samples of raw cotton may survive the manufacturing processes. It is believed that many less resistant bacteria may also survive.

Fleming and Thaysen[6] found cotton deterioration in damp storage to be caused by a streptothrix and a cellulose-decomposing *actinomyces*.

ALEXANDER, loc. cit.

[1] FARR, W. K., *Jour. Physical Chem.* **5** 228 1927

[2] FARR, W. K. and ECKERSON, H. *Contrib. Boyce Thompson Inst.* **6** 189 309 1934

[3] *PRINDLE, B. Text's Research* **4** 11 1934

[4] *P. cit.*

[6] *FLEMING, N. and J. L. THAYSEN, Biochem. Jour.* **14** 25 1920

Searle,[1] while investigating the rotting of textiles by microorganisms, found that *Stachybotrys* sp. was the most commonly occurring mold.
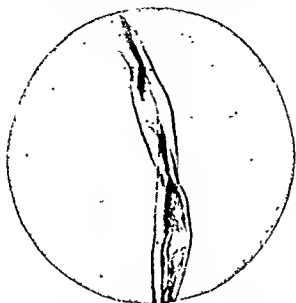
Fig. 113—Cotton fiber   *(Courtesy of The Textile Laboratory—Massachusetts Institute of Technology)*

Galloway[2] has described 180 molds isolated from injured fabrics. He states that raw cotton is a source of much of the infection of cloth, and that some of the infection, for example, *Aspergillus niger*, may come from the boll.

TABLE 172 —BACTERIAL AND MOLD CONTENTS OF SEED COTTON, RAW COTTON, AND STORED COTTON[1]

	Seed cotton, 4 samples	Raw cotton, 12 samples	Stored cotton, 5 samples
Bacteria per gram.			
Highest count	620,000,000	20,000,000	830,000
Lowest count	27,000,000	95,000	38,000
Average ..	220,000,000	7,900,000	67,000
Molds per gram:			
Highest count..	1,150,000	480,000	2,250
Lowest count	50,000	<3,000	65
Average.	565,000	109,000	687

[1] PRINDLE, B., *Textile Research*, **5**: 542 (1935).

According to Trotman and Sutton,[3] *Bacillus subtilis* and *B. mesentericus* may grow on cotton containing more than 9 per cent of moisture

[1] SEARLE, G. O , *Jour. Textile Inst.*, **20**: T162 (1929)
[2] GALLOWAY, L. D., *Jour. Textile Inst.*, **21**: T277 (1930).
[3] TROTMAN, S R., and R W. SUTTON, *Jour Soc Chem Ind*, **43**: T190 (1923)

and cause deterioration of the fibers. (Neither of these organisms is able to decompose cellulose.)

Bacteria may cause the destruction of fibers and fabrics, even though fungi are not present. When damp cotton is baled, anaerobic cellulose-decomposing bacteria are likely to become active, especially in the center of the bale.

Fig 114 —Fungus growth on cotton fiber *(Courtesy of The Textile Laboratory— Massachusetts Institute of Technology)*

Microorganisms may be picked up during the growth, harvesting, transportation, and manufacture of cotton Ginning and mechanical cleaning operations frequently do not materially reduce the numbers of microorganisms, and it is possible for mold spores to survive through the processes of spinning, sizing, and weaving

Resistance of Different Cottons to Deterioration.—Thaysen and Bunker have presented experimental data that indicate that cottons may vary considerably in resistance to microbiological deterioration. American cotton was most resistant, Egyptian cotton was less so, and India cotton decayed most rapidly. Thaysen and Bunker believe that

¹ THAYSEN, A. C , and H J BUNKER, *Biochem Jour* , 18: 140 (1924).

the difference in resistance was due at least partially to differences in
climate and soil conditions.

Fishing Nets.—Bacteria are responsible for a large percentage of the
damage to fishing nets. As a result of this destructive action, consider-
able research has been carried out by the U.S. Bureau of Fisheries and
other agencies to develop antiseptics to protect the nets from rotting.
Copper salts have been widely used as antiseptics for nets.

The Examination of Textile Fibers and Fabrics for Damage.—There
are several methods for examining textiles or textile materials for evi-
dences of damage. These include the use of the hand lens, the micro-
scope, culture media, tensile-strength tests, viscosity tests, and other
procedures. In conjunction with the use of the microscope, various stain-
ing agents have been used, alone and together with swelling reagents.

Color production, due to spores, perithecia, or pigments, or the musty
odor frequently makes it possible to distinguish fungus growth on fabrics
without more precise examination.

The microscope has given much valuable information[1] concerning
the structure and condition of fibers. The use of stains, such as Victoria
Blue B, in combination with swelling reagents has yielded particularly
good results.

Victoria Blue B Stain.[1] A 0.15 per cent aqueous solution of Victoria
Blue B is added to the sample of cotton in the proportion of approxi-
mately 15 cc of dye solution per 0.1 g of sample, an excess of dye thus
being assured. The sample is boiled for 1 min. in the dye, washed with
cold water until no further color is removed, boiled with distilled water
until bleeding of the color ceases, rinsed with cold water, permitted to
drain, and then dried on a piece of filter or blotting paper.

Swelling Tests. There are three swelling tests that have often been
used to determine details of the structure of cotton as well as to obtain
evidence of damage. These include the carbon bisulphide-sodium
hydroxide test of Fleming and Thaysen,[2] the cuprammonium test, and
the "critical" sulphuric acid test.

THE SWELLING TEST OF FLEMING AND THAYSEN.—A uniform sample of
the fibers is prepared by first carefully mixing 3 g. of the material. To
approximately 0.2 g of the sample are added 10 cc. each of carbon
bisulphide and of 15 per cent sodium hydroxide solution. The sample
and chemical reagents are shaken occasionally, while soaking of the

[1] BARRITT, T. B., *Jour. Roy. Microscop. Soc.*, p. 141 (1925).
[2] PRINDLE, B., *Textile Research*, 6: 481 (1936).
[3] FLEMING, N., and A. C. THAYSEN, *Biochem. Jour.*, 15: 407 (1921).
[4] THAYSEN, A. C., and H. J. BUNKER, "The Microbiology of Cellulose, Hemi-
celluloses, Pectin and Gums," Oxford University Press, New York, 1927.

fibers or hairs proceeds until the required swelling has been obtained (ascertained by examining small samples microscopically from time to time to determine when the swelling has reached an optimum point). Then three samples, each about the size of a large pea, are placed on three glass slides. The sample on each slide is mixed carefully, and approximately 20 fibers, selected at random, are "spread out horizontally" and covered with a cover glass, a drop of water being permitted to diffuse under the cover slip. The slide is examined microscopically. By scrutinizing three or more slides and making 10 counts on each slide for sound and damaged fibers, this test may be made quantitative in nature.

Normal cotton fibers present a beaded appearance when treated with swelling reagents. This result is due to the fact that the cuticles offer resistance to the expansion of the cellulose layers enclosed by them, while no resistance is offered to the layers between the cuticles. In damaged cotton hairs the cuticle has been injured or destroyed, while the cellulose has undergone some change. Consequently the beaded appearance found in normal cotton hairs is missing.

THE CUPRAMMONIUM SWELLING TEST [1]

When this test is to be made, it is desirable to have all the necessary equipment close at hand and prepared. Forceps, needles, clean glass slides, cover slips, cuprammonium solution, and microscope should be available.[1]

Fig. 115 Cotton fiber swollen in sodium hydroxide and pressure (Courtesy of The Textile Laboratory—Massachusetts Institute of Technology)

The cuprammonium solution is prepared in the following manner: In 100 cc. of distilled water are dissolved 15.7 g. of copper sulphate ($CuSO_4 \cdot 5H_2O$). About 25 cc. of 6 N sodium hydroxide are added to this solution in order to precipitate the copper as copper hydroxide. The copper hydroxide mixture is filtered, and the hydroxide is freed of the sulphate ion by washing it with water. Finally the washed precipitate of copper hydroxide is dissolved in 500 cc. of 15.5 per cent ammonium hydroxide, and the resultant solution is stored in a brown glass bottle, which should be kept tightly stoppered.

[1] Prindle, B., *Textile Research*, 6: 481 (1936).

The sample to be examined, which may be previously stained with Victoria Blue B and dried according to the procedure of Dr. Prindle, is slightly dampened by breathing onto it. It is placed on the top surface of a clean glass slide that has also been breathed upon (reducing the tendency of the fibers to fly about). A few of the fibers are pulled from the sample with the forceps and placed parallel to the long edge of the slide.

A few drops of cuprammonium solution from a partly filled bottle are added to the fibers, and a cover slip is placed over them. The mount is examined with a 100 × magnification. The cuprammonium solution will be too concentrated for most samples of cotton. Therefore small quantities of distilled water are added to the bottle containing the cuprammonium solution to reduce the concentration. The additions should be made gradually, the effect of a given concentration of the solution on the swelling rate being observed after each addition. In this manner, the proper dilution of cuprammonium solution for the sample being examined may be determined.

The correct concentration having been discovered, the fibers (placed in a parallel position on the slide) are covered with a few drops of the solution, and a cover slip is placed over the fibers plus the solution in such a manner that no air bubbles will be entrapped. The rate and nature of the swelling should be observed immediately with the microscope.

Normal samples of undamaged raw cotton are usually stained to an even dark purple-blue by the Victoria Blue B, according to Prindle, while mildewed samples exhibit a mottled appearance with "a lighter and clearer blue." It is believed that this color change is due to the fact that the pH of the dye has been altered by the acid nature of the mildewed area. This difference in the staining by Victoria Blue B is very helpful in distinguishing mildewed spots on fiber from normal fiber.

The swelling of the fibers is similar to that which takes place in the Fleming and Thaysen test, i.e., normal fibers demonstrate a beaded appearance at a low magnification of the microscope.

Cuprammonium solution may be prepared readily and stored for long periods without loss of value. It is easy to control the concentration of the solution which is directly related to the rate of swelling of the sample.

The combination of staining with Victoria Blue B and the use of the cuprammonium reagent reveals much information concerning the condition of the fiber and details of the material contained in the lumen and the cuticle.

Research carried out by Kusebauch[1] using the Victoria Blue B stain

[1] KUSEBAUCH, K., Melliand Textilber., 17: 18 (1936).

followed by the use of the cuprammonium reagent confirms the observations made independently by Prindle.

Safranine-methylene Blue Stain for Mold Filaments in Cotton Fibers.—The sample of cotton is wetted with 95 per cent ethanol, stained for 1 min. with Loeffler's alkaline solution of methylene blue, washed with water, counterstained with safranine for 10 sec. or less, washed with water, dried, and examined with the low and high magnification of the microscope.

Pianese IIIb Stain [1].—For the detection of mold mycelium in mildewed cotton fabrics, Jennison recommends the use of Pianese IIIb as a differential stain. The sample is washed in 95 per cent ethanol, stained for 15 to 45 min. with Pianese IIIb, washed with water, decolorized with acid alcohol, mounted on a glass slide, and examined microscopically.

The Congo Red Test.—The Congo red test may be used to estimate the damage caused to fibers by microorganisms, heat, acid, and mechanical means.

The Congo red test is as follows: Approximately 0 1 g of cotton is put into a suction flask with water. As much of the air as possible is evacuated. The cotton, after thus being wetted, is gently pressed to get rid of most of the water It is placed in 25 cc of an 11 per cent sodium hydroxide solution, shaken carefully, and permitted to stand for 5 min The sample of cotton is quickly washed in water, placed in a saturated solution of Congo red (about 2 per cent), shaken intermittently for 6 min, removed, and washed with water until the water no longer shows any pink color. It is placed in an 18 per cent solution of sodium hydroxide. A few hairs are teased from the sample and mounted in the hydroxide solution on a glass slide They are examined microscopically

Undamaged cotton hairs are stained pink Cotton hairs damaged by fungi are stained red and may be cracked. Such damage is nearly always associated with the presence of at least traces of the organism Mechanically damaged hairs show bruises and cuts stained deeply. Hairs damaged by heat may show red spiral bands and singed cuticles Injury caused by sulphuric acid results in irregular patches stained red

Test for Mildew Resistance of Outdoor Cotton Fabrics [2].—This test is designed to estimate the efficiency of the processes used to increase mildew resistance of cotton fabrics for outdoor use *Chaetomium globosum* was selected as the test organism principally on account of its very

[1] JENNISON, M W, *Science*, **72**: 316 (1930).
[2] BRIGHT, T B, *Jour Textile Inst*, **17**: T396 (1926)
[3] THOM, C, H HUMFELD and H P HOLMAN, *Am Dyestuff Rpt*, Oct 22 1931

destructive action on cotton fabric, its ease of cultivation, and its ability
to grow under a variety of conditions. The test is as follows:

A sample of the fabric, sufficiently large to yield 12 strips, each 4 in.
long in the direction of the warp and 1.25 in. wide, is soaked in running

FIG. 116.—Typical appearance of mildewed fiber under dissecting objective, showing
general absence of strictures caused by resistant cuticle and the bunching of fibers which
were parallel before swelling   [Courtesy of B Prindle, Textile Res., 6: 481 (1936)]

FIG. 117.—Appearance of mildewed fiber under 16-mm objective showing absence of
. . .   The

water, or in changes of water, for 2 days in order to extract water-soluble
antiseptics that would be removed normally by rain or other weather
conditions. The sample is air dried and cut into at least 12 strips of the
dimensions stated. Five of these strips, which are to serve as controls,
are put into one bottle, the remainder into a second bottle. The bottles

with their contents are sterilized in an autoclave at a pressure of 15 lb per sq. in. for 30 min. After cooling, the strips are ready for the test

A special agar medium is prepared, which contains 3 g. of sodium nitrate; 1 g. of dipotassium hydrogen phosphate, 0 25 g. of sodium chloride; 0.25 g. of magnesium sulphate ($MgSO_4.7H_2O$); 0 01 g. ferrous sulphate; 10 g. of agar, and 1,000 cc. of distilled water The pH of the medium is adjusted to 6 8 and autoclaved for 20 min at a pressure of 15 lb

Agar is poured to a depth of 3 mm. in sterilized petri dishes that are 20 mm in diameter and 15 mm deep At least seven plates are required for a test.

The sterile strips of cotton fabric may be infected with the spores of *Chaetomium* in one of two ways They may be shaken in a sterile container with 10 cc of a spore suspension, and then placed, one each, in separate petri dishes containing agar, using aseptic technique In the alternate method, 1 cc of the spore suspension is evenly distributed over the surface of the solidified agar in a petri dish, and then a strip is aseptically placed over the infected agar.

Since the agar medium contains no assimilable carbon, the cellulose of the fabric must serve as the source of energy

The plates are incubated for 14 days at a temperature of 28 to 30°C At the end of this period, the five strips that exhibit the most even covering with mold growth are washed to free them of agar and mold, and air dried.

The strips that have been subjected to mold action and the controls are raveled down in order that each strip may contain the same number of threads, corresponding to 1 in. of the original sample (a thread counter is advocated for this purpose) These strips are then placed in a chamber of constant temperature and humidity, for 2 days, in order to obtain results comparable with other tests carried out at various other times The tensile strength of the fabric is then determined by a standard apparatus

WOOL

Structure.—The wool fiber contains two principal layers of cells: the scales on the exterior and the cortex on the interior Occasionally there is a trace of a medullary layer, which is always present in the hair fiber The scales overlap one another, similar to those of a fish, and usually are highly serrated and loosely attached Scales of hair are set more tightly to the cortex. Softness, ability to felt, and luster are related to the arrangement and kinds of scales They impart strength and elasticity to the fiber, while and elongated The cortical cells are fibrous the size and nature of these cells govern the waviness of the fiber

Chemical Composition.—Chemically wool is made up largely of proteins. Keratin, which is found in hair, nails, hoofs, and horns, is the principal one. The average chemical composition varies with the type

of animal, food, etc. On hydrolysis, keratin yields at least 16 different amino acids, of which glutamic, leucine, arginine, and aspartic acids are present in relatively large quantities. Sulphur is present to the extent of about 3.5 per cent in average wool, being a constituent mainly of cystine.

Speakman, Goddard and Michaelis, Ashbury, and others have studied the structure and composition of the wool fiber.[1]

Several substances are usually found deposited on the surface of the wool fiber. These include wool greases (compare with lanolin); water-soluble compounds, such as potassium soaps and potassium salts, urea, etc.; soil; and other matter. The matter thus found on wool is known as "yolk."

Microbiology.—The microbiology of wool has been investigated by various workers. Burgess, Trotman and Sutton, Bright, Galloway, Prindle, and others are outstanding in their contributions to this phase of textile science

Molds, actinomycetes, bacteria, and yeasts have been found on wool and may bring about undesirable changes, which include discolorations and deterioration of the fibers.

Prindle[2] has isolated, among the molds, species of *Alternaria*, *Stemphylium*, *Oöspora*, and *Penicillium* that completely or partially destroy the structure of wool Species of *Alternaria*, *Stemphylium*, and *Oöspora* were particularly destructive and productive of discolorations. Other molds capable of altering the structure of wool include species of *Aspergillus*, *Dematium*, *Fusarium*, *Trichoderma*, and *Cephalothecium*.

Of the bacteria, several aerobic bacilli have been found to have the

[1] SPEAKMAN, J B , *Jour. Soc. Dyers Colourists*, Jubilee Issue, 1934, GODDARD, D. R., and L MICHAELIS, *Jour. Biol. Chem* , 106: 605 (1934), ASHBURY, W. T., *Nature*, 140: 968 (1937).

[2] PRINDLE, B., *Textile Research*, 5: 542, 6: 23 (1935).

ability to deteriorate wool, while a few cocci caused discolorations (pink to red), without injuring the strength of the fiber. Nonsporeforming rods also have produced discolorations, which may be red, yellow, orange, or of other color. The alkaline nature of fleece favors the growth of some types of bacteria rather than that of molds

The scouring and drying of wool during processing removes or inactivates a large part of the molds and nonsporeforming bacteria, but aerobic bacilli survive.

Sterilization of Wool.—Humfeld, Elmquist, and Kettering[1] have studied several methods for sterilizing wool fabrics in an effort to determine the most satisfactory one for use in making subsequent bacteriological studies, using *Bacillus mesentericus* as the test organism. They found that wool fabrics could be heated in xylene, Stoddard solvent, or tetrachlorethylene satisfactorily, spores being destroyed and the fabrics being undamaged The method consisted of heating the fabrics with xylene for 12 hr. at 100°C', or for 2 hr at 121°C , or for 1 hr at 134.5°C. No bacteriostatic effects were noticed in fabrics thus treated

Physical and chemical properties of wool were altered by the use of intermittent steaming and wet or dry autoclaving ?

Ultraviolet light, iodine, potassium permanganate, alcohols, and certain other agents failed to produce sterility of the wool under the conditions of the experiment.

Formaldehyde and mercury salts produced sterility of the fabrics but were retained to some extent

For further details consult the bulletin cited

Sieber's Test for Damaged Wool.—A sample of wool is degreased by washing it first in ether, then in water The washed sample is boiled for a few minutes in a 1 per cent solution of Benzopurpurine 10 B The dyed sample is boiled with water until no more color is extracted It is then examined with a microscope. Those parts of the wool which have been damaged mechanically by acid or alkali will be stained red

Fiber vs. Fabric.—The foregoing pages have dealt with fibers most commonly used in textile manufacturing In making and finishing many types of the fabrics now on the market two or more classes of fibers may be employed, and, furthermore, the woven goods may be subjected to a variety of processes such as sizing (with starchy materials), bleaching, dyeing, and use of finishers These processes may exert an effect on the microbial populations attached to the fibers or may change the life-supporting character of the finished goods From this standpoint,

¹ HUMFELD, H R : ELMQUIST, and I H KETTERING, U S Dept Agr , Tech Bull 588, September, 1937
² Ibid

probably the most important of the processes named is the use of sizing materials, which in themselves may introduce new infection with microbes, and which are not always completely removed by the diastatic preparations employed as desizing agents.

The Prevention of Growth of Microorganisms on Textiles.—There are two principal methods for preventing the growth of microorganisms on textile fibers and fabrics The first and the only sure method (thus far known) consists of reducing the moisture content of the goods to less than 8 per cent and maintaining it below this maximum. Galloway[1] has shown the importance of relative humidity in relation to the storage of fabrics. He stated that the "safe" figure for storage depended on the microorganism instead of the nature of the material. Thus the minimum relative humidity that permitted the growth of several types of molds varied between 75 and 95 per cent. Certain species of *Aspergillus*—for example, *A glaucus, A. candidus*, and *A. versicolor*—were able to grow at relative humidities of 75 to 80 per cent. Thus, in order to prevent mildew it is necessary to maintain a relative humidity that is too low to permit growth.

Obviously it is impossible to keep dry such articles as tarpaulins, tents, and fish nets. The second method indicates the use of a suitable antiseptic. Although fairly satisfactory chemical agents have been tried as textile antiseptics, the search for the ideal antiseptic still continues Many substances have been proposed, as is evidenced by the large number of publications dealing with this subject.

Morris and others[2] have outlined the properties of the good antiseptic. The good antiseptic (1) must be sufficiently soluble in water to mix evenly with the size; (2) must be stable to heat (for example, during the boiling of the size), drying, and oxidation; (3) should be odorless at various pH values; (4) should be free of color and should not alter that of the fabric; (5) must not injure the fabric; (6) must not injure metal attachments or machinery; (7) must not affect dyeing and finishing operations; (8) must not alter the size and thus affect the "feel" of the fabric; (9) must be safe to handle, and (10) must be readily available in large quantities and at a low cost.

Tarpaulins, tent canvas, awnings, roofing paper, and fishing nets may be preserved from the action of microorganisms by the application of antiseptics. Copper compounds have been widely used, especially to treat fish nets. Copper oleate, as well as copper oxide and mercuric

[1] GALLOWAY, L D , *Jour. Textile Inst*, 26: T123 (1935).

[2] MORRIS, L E , *Jour Textile Inst*, 18: T99 (1927), FARGHER, R G , L D. GALLOWAY, and M E ROBART, *Jour. Textile Inst*, 21: T245 (1930), ROBERTS, H C., *Am Dyestuff Rpt* , 19 : 431 (1930)

oxide, or mixtures of these, have been recommended by Conn.[1] Copper 8-quinolinolate, copper naphthenate, copper ammonium fluoride, zinc naphthenate, dihydroxydichlorodiphenylmethane, pyridylmercuric stearate, and salicylanilide (Block, 1946) are useful textile fungicides.

According to Taylor and Wells,[2] copper oleate is particularly effective when applied a second time to a fishing line that has been immersed for some time after the first treatment Copper compounds add color to the fabrics being treated, and for this reason their field of application is limited

Zinc chloride has been used extensively in sizes, but it is a heavy compound and not desirable for certain fabrics Benzoates, borates, complex fluoride salts, salicylates, and many other chemical compounds have been used with variable results.

Oil of thyme, particularly when mixed with turpentine and rosin oil, has been used as a disinfectant for carpets (Funch-Hellet), and for other purposes.

Galloway has recommended the use of *p*-chloro-*m*-cresol for finishing baths He has suggested also (1930) the use of carbon dioxide as a storage gas

Proctor has shown that fibers inoculated with *Aspergillus niger* and then treated with a mixture of ethylene oxide gas and carbon dioxide did not evidence mold development when subsequently exposed to the air. This suggests that storage of finished fabrics in chambers provided with these inhibitory gases may be commercially applied

For an extensive review of this subject, the student is referred to the textile journals, patent literature, and other publications. The reports by Block (1916), Lesser (1947), and the Quartermaster Corps are particularly significant

RETTING

Purpose.—The fibers of flax and hemp (bast fibers) are commonly loosened from the stems that contain them by a process known as "retting" This is an ancient term meaning "soaking in water" The fiber bundles of flax lie between the soft-walled cells[3] of the cortex and of the central wood Retting, if efficient, should accomplish a satisfactory loosening of the fiber bundles from the cortex and wood and effect a partial digestion or loosening, at least, of the cementing material between the various fiber bundles

[1] Conn, W T, *U S Bur Fisheries Doc*, 1075, 1930
[2] Taylor, H P and A F Wells, *U S Bur Fisheries Docs* 947 (1923) and 998 (1925)
[3] Lind, J V, and C R Nodder, *Jour Textile Inst*, 15: 7237 (1921)

Methods.—Retting is accomplished by both anaerobic and aerobic methods. Anaerobic methods include those in which retting is carried out in flowing or in stagnant waters. For example, the Egyptians have for hundreds of years retted flax in the soft, warm, slow-flowing waters of the River Nile under almost ideal conditions. Retting in slow-flowing waters has also been practiced in Belgium, Germany, and Holland; while Italy and Ireland have made use of stagnant waters, such as ponds and dams. Aerobic methods may include those in which the material is retted in vats supplied with aeration or the method known as the "dew" or "land-retting" method.
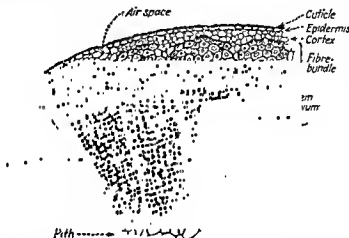
FIG. 119 —Diagrammatic cross section of a flax stem. [*Courtesy of Eyre and Nodder, Jour Textile Inst.,* **15** : T239 (1924)]

Retting is accomplished in the anaerobic methods principally as the result of bacterial action, in the aerobic dew method, largely by the action of fungi.

Selection of Method.—The choice of method will depend on several factors. the agricultural situation, the nature and quantity of the available straw, the water supply, the climate, the costs involved, and other factors.

Anaerobic Retting.—The following comments will be confined to a general discussion of retting as carried out by anaerobic processes, whether in a vat, a river, or other place.

Retting may be considered to take place in three stages: a physical stage; a biological stage, which may be subdivided into preliminary and principal phases; and a mechanical stage.[1]

1. *Preliminary.*—It is important to prepare the material carefully for retting. For example, in the deseeding of the flax plant, damaging of the fibers leads to the evils accompanying overretting, *i.e.*, weakening,

[1] THAYSEN and BUNKER, *loc. cit.*

discoloration, etc. The raw material should be carefully sorted and treated according to its nature and condition.

The chemical changes that take place will vary according to the types of microorganisms that predominate, the nature of the water, the material being retted, and the method used.

2. *Physical Stage* —During the physical stage, water is absorbed by the tissues of the stem, swelling occurs, water-soluble substances are extracted, and bacteria develop Fissures and breaks frequently appear in the woody portion, while bubbles of air are given forth from the tissues The substances extracted, which amount roughly to 12 per cent, include sugars, glucosides, tannins, soluble nitrogenous constituents, and coloring matter The surrounding liquid thus becomes a highly colored medium for the development of bacteria and other microorganisms.

3. *Biological Stage* —During the preliminary biological stage, many of the bacteria and other microorganisms, which are resident on the materials being retted, grow and multiply. Aerobic forms predominate at first, since the water contains dissolved oxygen and nutrients favoring their development Yeasts and molds may develop on the surface of the water. In using up the dissolved oxygen, the aerobic organisms tend to establish anaerobic conditions. Organic acids and gases, especially carbon dioxide, are produced.

Actually retting occurs mainly during the principal biological stage. The middle lamella is softened, cells of the plant tissues are separated, and the connections between the bundles become weakened.

The anaerobic organisms responsible for retting multiply rapidly during this period These include the bacteria, which elaborate the enzymes capable of hydrolyzing the pectin of the middle lamella of the parenchymatous bark tissue, thus causing a separation of the fiber bundles from the cortex and wood *Plectridium pectinovorum* (related to *Clostridium butyricum* Prazmowski[1]) and *Cl. felsineum* (*Bacillus felsineus*) are two anaerobic pectin-dissolving bacteria of considerable value in retting processes. Ruschmann[2] considers *B. amylobacter* (also related to *Cl butyricum* Prazmowski) to be the most important anaerobic organism in the warm-water vat process Makrinov[3] used pure cultures of *Pectinobacter amylophylum* with "superior retting results"

Various substances are produced during the fermentation, depending on the organisms and the conditions of the retting operation These may include organic acids, such as acetic and butyric acids, gases—carbon

[1] "Berges's Manual of Determinative Bacteriology," p 770, 6th ed , The Williams & Wilkins Company, Baltimore, 1948

[2] Ruschmann, G , *Jour Textile Inst* , **15**: T61, T104 (1924)

[3] Makrinov, I A , Dnevnik Vsespuznogo, *Sci Botan Leningrad*, 206: 1928 (1928)

dioxide, hydrogen, and sometimes methane and hydrogen sulphide; solvents, such as ethanol, butanol, and acetone; and other substances. Lactic acid production interferes with the action of butyric acid bacteria, such as *B. amylobacter*. Organic acids may be oxidized at the surface of the vat to simpler compounds.

It is important not to overret flax, since the liquified pectin of the fiber bundles may be hydrolyzed with the result that individual fibers become separated.

Cellulose is not fermented by desirable retting organisms.

4. Mechanical Stage.—The retted material is washed (if in a vat, by an upward flow of water) to carry away portions of mold films, organic acids, odors, and other undesirable substances that may be associated with the plant material. Sometimes just sufficient alkali, in the form of soda, is added to neutralize the acids present in the vat (if one is used), resulting in an increase in the luster and suppleness of the fiber. Another method for freeing the material from organic acids after washing is to expose it to the action of aerobic bacteria.

The washed material is carefully dried by natural or artificial means.

Finally, the dried retted fiber tissue is separated from the cortical and wood residues by the use of machines.[1]

Temperature of Retting.—A fairly wide range of temperatures has been used in various retting processes. In general, it may be stated that higher temperatures, 37 to 38°C., for example, favor rapid retting but are sometimes less desirable from the viewpoint of the quality of the final product, for overretting is more likely to occur at higher temperatures and leads to the damaging of the fibers. Good results may be obtained, however, by carefully controlling the processes that use higher temperatures. The Carbone retting process employs a relatively high temperature.

Temperatures of 26 to 28°C. or 30 to 32°C. are considered to be most favorable for retting.[2]

Dew retting is subject to wide variations of the temperature.

Carbone Retting Process.—In this process, a mass culture of *Cl. felsineum* (*B. felsineus*) is prepared in a potato medium and added at the rate of 1 liter to 10 kg. of dry tissue to the water of the retting vat. The retting temperature is 37 to 38°C., the optimum for the growth of this organism. A period of 50 hr. or less is usually required for retting, but a longer time may sometimes be necessary. This process naturally requires closer supervision than certain other retting processes. The fibers produced are bright colored, while the yield is said to be good.

[1] THAYSEN and BUNKER, *loc. cit.*
[2] RUSCHMANN, *loc. cit.*

A résumé of investigations on the water retting of flax during the period of 1940 to 1945 has been published by the Council for Scientific and Industrial Research, Commonwealth of Australia (Greenhill and Couchman, 1947).

Aerobic Retting Processes.—An aerobic method of retting was developed by G. Rossi. A mass culture of *B. comesii* is added to the plant material in a vat. The water, maintained at 28 to 30°C, is aerated to favor the development of aerobic bacteria, *B. comesii* in particular. It has been stated that a smaller quantity of organic acid is produced by this process, while the danger of overretting is greatly reduced and fibers may be dried artificially without danger. According to Ruschmann, such fibers are fuller in appearance, darker, and harder.

Dew Retting.—The retting action of this process is due principally to molds, but bacteria are present in large numbers.

In carrying out the process, the material to be retted is spread out in thin layers on suitable vegetation. It is thus exposed to the action of the sun, dew, and rain. Atmospheric conditions, the retting bed, and the soil are mainly responsible for the quality of retting. As would be expected, there is a minimum of control used in this process, which is simple and inexpensive. Although good fiber may be obtained by this method, the fibers are frequently of a poor quality and the yields small.

Improvements in Methods.—In this very brief description of a limited number of retting methods, it has been impossible to discuss adequately the problems of the processes, which are concerned with the yield of the fiber and its quality—softness, strength, color, etc. It will suffice to state that improvements in procedures are constantly being made. A review of the literature of textile journals will yield information along these lines to the zealous student.

Periodicals on Textile Microbiology and the Textile Industry

American Dyestuff Reporter (fortnightly), Howes Publishing Co., New York.

Bulletin of the U.S. Institute for Textile Research, Inc. (monthly), Boston.

Canadian Textile Journal (weekly), Canadian Textile Journal Publishing Co., Ltd., Montreal.

Cotton (weekly), Manchester Cotton Association, Ltd., Royal Exchange, Manchester, England.

Cotton (monthly), W. R. C. Smith Publishing Co., Atlanta, Ga.

Deutsche Kunstseiden Zeitung und Spezialorgan für Zellwolle (semiweekly), Berlin.

Faserforschung. Zeitschrift für Wissenschaft und Technik der Faserpflanzen und der Bastfaser-industrie (irregular), S. Hirzel, Leipzig.

Indian Textile Journal (monthly), Indian Textile Journal, Ltd., Bombay.

Journal Textile Institute (monthly), Manchester, England.

Kunstseide und Zellwolle (monthly), H. Jentgen-Verlag, Berlin.

Melliand Textilberichte (monthly), Heidelberg.

National Canvas Goods Manufacturers Review (monthly), National Tent and Awning
 Manufacturers Association, St Paul, Minn.
Rayon Textile Monthly, Rayon Publishing Corp., New York.
Rayon World (monthly), Osaka, Japan.
Silk and Rayon, Journal Devoted to the Progress of the Silk and Rayon Industries
 (monthly), Manchester, England.
Textile Colorist, Devoted to Practical Dyeing, Bleaching, Printing and Finishing
 (monthly), Textile Colorist, Inc., New York.
Textile Manufacturer (monthly), Manchester, England.
Textile Research (monthly), U.S Institute for Textile Research, Inc., Boston.
Textile World (monthly), McGraw-Hill Publishing Company, Inc., New York.
Wool, Review of the World's Wool Industries (monthly), London.
See also periodical directories.

References on Textile Microbiology and Textiles

ALEXANDER, J : Textile Fibres—Chemical and Physical Aspects, *Ind. Eng. Chem*,
 31: 630 (1939).

ANON.: Cuticle of Cotton, *Textile Mfr.*, p. 354, September, 1936

ASHBURY, W. T : "Fundamentals of Fibre Structure," Oxford University Press, New
 York, 1933.

BARKER, S. G : "Wool Quality," H. M. Stationery Office, London, 1931.

BARLOW, C M : A Method of Embedding Fibres for Section Cutting in a Medium
 Containing Ethyl Cellulose, *Jour. Textile Inst*, **29**: T111 (1938).

BENIGNUS, P G : Copper 8-quinolinolate Industrial Preservative, *Ind. Eng Chem*,
 40 (No 8): 1426–1429 (1948)

BLOCK, S S : Mold and Mildew Control for Industry and the Home, *Florida Eng Ind.
 Expt Stat*, *Bull.* 12, University of Florida, November, 1946

BORGHETTY, H C : Lanital, the Artificial Wool made from Casein, *Am. Dyestuff
 Rptr*, **25**: 538 (1936).

BRANFOOT, M H : A Critical and Historical Study of the Pectic Substances of Plants,
 Great Britain Food Investigation Board, Special Report 33, H M. Stationery
 Office, London, 1929

BRIGHT, T B : Methods of Examination of Mildewed Cotton Material, *Jour Roy
 Microscop Soc* p. 141, 1925
——: The   d
 Test and   )6
 (1926)
——, L. E MORRIS, and SUMMERS, F : Mildewing in Cotton Goods, *Jour. Textile
 Inst* , **15**: T547 (1924)

BURGESS, R : Studies on the Bacteriology and Mycology of Wool, *Jour Textile Inst*,
 15: T573 (1924).
——: A Contribution to the Study of the Microbiology of Wool, *Jour. Textile Inst*,
 19: T315 (1928).
——: Microbiology of Wool. The Enhancement of "Mildew" by Soaps and
 Vegetable Oils   *Jour Textile Inst* **20**: T333 (1929)
——: Mildew Pro
——: The Use of
 Bacterial Disintegration, *Jour Textile Inst*, 20
——: Causes and Prevention of Mildew on Wool, *Jour. Soc. Dyers Colourists*,
 138 (1934)

────── and C. RIMINGTON: A Technique for the Microscopical Examination of Wool Fibres, *Jour. Roy. Microscop. Soc.*, 49: 341 (1929).

CARBONE, D., and A MOGGI: *Boll. sez. stal., Soc intern. microbiol.*, 7: 30 (1935).

CARSWELL, T. S, and H K NASON: Properties and Uses of Pentachlorophenol, *Ind. Eng. Chem.*, 30: 622-626 (1938).

CERIGHELLI, R.: *Agron. Colon*, 23: 33 (1934).

CHACE, W. G.: Microbiology of Textiles: Review of Recent Progress in, *Am. Dyestuffs Rptr.*, 26: 652 (1936).

CONN, W. T.: Net Preservation Treatments (Appendix to Report of Commissioner of Fisheries for the Fiscal Year 1930), *U S Dept. Comm*, *Bur. Fisheries Doc.* 1075, 1930.

COOLHAAS, C., Zur Kenntnis der Dissimilation fettsaurer Salze und Kohlenhydrate durch thermophile Bakterien II Die Dissimilation von Stärke und Zuckerarten durch thermophile Pakterien, *Centr Bakt. Parasitenk*, Abt. II, 75: 344 (1928).

────── III. Die Dissimilation von Zellulose durch thermophile Bakterien. *Centr. Bakt Parasitenk*, Abt II, 76: 38 (1928)

"Directory of Newspapers and Periodicals 1938," N. W. Ayer & Son, Inc., Philadelphia, 1938

ELMQUIST, R. E, and O F HARTLEY Methylene Blue Absorption as a Quantitative Measure of Wool Damage, *Textile Research*, 5, 149 (1935)

EYRE, J V, and C R NODDER An Experimental Study of Flax Retting. 1. The Development of Acidity during the Retting of Flax. Its Interpretation and Technical Significance, *Jour Textile Inst*, 15: T237 (1924)

FARGHER, R G, L D GALLOWAY, and M E ROBART The Inhibitory Action of Certain Substances on the Growth of Mold Fungi, *Jour Textile Inst*, 21: T245 (1930)

FARR, W K Structure of the Cotton Fiber, *Jour. Applied Phys.*, 8: 228 (1937).

────── and S H ECKERSON *Contrib Boyce Thompson Inst*, 6: 189, 309 (1934)

"First Report of the Fabrics Coordinating Research Committee," Dept of Scientific and Industrial Research, H M Stationery Office, London, 1925

FLEMING, N, and A C THAYSEN On the Deterioration of Cotton on Wet Storage, *Biochem Jour.*, 14: 25 (1920)

────── and ────── The Deterioration of Cotton on Wet Storage, *Biochem Jour*, 15: 406 (1921).

FUNCH-HELLET *Jour Soc Dye Colourists*, 52: 142 (1936)

GALLOWAY, L D The Fungi Causing Mildew in Cotton Goods, *Jour Textile Inst*, 21: T277 (1930)

────── The Moisture Requirements of Mold Fungi with Special Reference to Mildew in Textiles, *Jour Textile Inst*, 26: T123 (1935)

GODDARD, D R, and L MICHAELIS A Study on Keratin, *Jour Biol Chem*, 106: 605 (1934)

GREENHILL, W L., and J F COLEMAN The Water Retting of Flax, *Council for Sci Ind Research, Bull* 211, Commonwealth of Australia, Melbourne, 1947

HALL, A J Rapid Methods of Detecting "Damage" in Cotton and Wool, *Am. Dyestuffs Rptr*, 2: 105, 139 (1932)

HUMFELD, H, R E ELMQUIST, and J H KETTERING The Sterilization of Wool and Its Effect on Physical and Chemical Properties of a Wool Fabric, *US Dept. Agr*, *Tech Bull* 588, September, 1937

JENNISON, M W. Detection of Fungus Mycelium in Mildewed Cotton Fabrics, *Science*, 72: 316 (1930)

KETTERING, J. H.: A Modified Method for Measuring Wool Damage Due to Scale Breakage, *Jour. Home Econ.*, **28**: 255 (1936).

KÜSEBAUCH, K.: Beitrage zur Anatomie der Baumwollfaser, *Melliand Textilber.*, **17**: 18 (1936). (Illustrated with colored photomicrographs.)

LA PIANA, F. G.: Sizing and Finishing of Textile Fabrics, *Textile Research*, **6**: 278 (1936).

LESSER, M. A.: Mildew Preservatives, *Soap Sanit. Chemicals*, **23** (No. 11): 119–121, 123, 152C, 153.

LEVINE, S., and F. P. VEITCH: Testing the Mildew Resistance of Textiles, *Jour. Ind. Eng. Chem.*, **12**: 139 (1920).

MAKRINOV, I. A.: *Dnevnik Vsesprunogo Sez Botankov. Leningrad*, **206**: 1928 (1928).

MATTHEWS, J. M.: "The Textile Fibres," John Wiley & Sons, Inc., New York, 1924.

MORRIS, L. E.: Mildew in Cotton Goods. Antiseptics and the Growth of Mold Fungi on Sizing and Finishing Materials, *Jour. Textile Inst.*, **18**: T99 (1927).

OSBORNE, G.: Micro-analysis of Textile Fibers, *Textile Research*, **4**: 84 (1934); **5**: 75, 275, 307, 351 (1935).

PRATT, F. C.: Wool and Mildew, *Textile Recorder*, **51**(609): 50 (1933).

PRESTON, J. M.: "Modern Textile Microscopy," Emmott & Co., Manchester, 1933.

———: Section Cutting by a Modified Plate Method, *Jour. Textile Inst.*, **27**: T216 (1936).

PRINDLE, B.: Microbiology of Textile Fibres. I. Study of Literature; Development of Methods, Qualitative Results, *Textile Research*, **3** (No. 10): 475 (1933).

———: The Microbiology of Textile Fibres. II. Cotton Fibre. *Textile Research*, **4** (No. 9): 413, **4** (No. 10): 463, **4** (No. 12): 555, **5** (No. 1): 11 (1934).

———: The Microbiology of Textile Fibres. III. Raw Cotton. IV. Raw Wool *Textile Research*, **5** (No 12): 542, **6** (No. 1): 23 (1935).

———: Microbiology of Textile Fibres. V. Method for the General Histological Examination of Normal or Mildewed Cotton Fibres, *Textile Research*, **6** (No. 11): 481 (1936).

———: Microbiology of Textile Fibres. VI. Relative Humidity over Certain Aqueous Solutions of Glycerol at 25°C., *Textile Research*, **7** (No. 11): 413 (1937).

———: Microbiology of Textile Fibres. VII. Growth of Mildew Organisms in Raw Cotton at 25°C and Relative Humidities of 70 to 90 Percent, *Textile Research*, **7** (No. 12): 445 (1937).

PROCTOR, B. E., and W. G. CHACE. "Textile Microbiology," in "Textile Research: A Survey of Progress," Massachusetts Institute of Technology, Cambridge, 1932

Quartermaster Corps: Methods for Analysis of Fungicides, Group XI. Textile Materials, Jefferson Quartermaster Depot.

RIMINGTON, C.: A Quantitative Method for Determination of "Soundness" in Wool and Cloth, *Jour. Textile Inst.*, **21**: T237 (1930)

ŘÍPA, R.: "Die Petinstoffe, Einfuhrung mit besonderem Hinblick auf die Obstkonserven-Industrie," Serger & Hempel, Braunschweig, 1937.

ROBERTS, H. C.: Mildew in Textile Mills, *Am. Dyestuff Rptr.*, **19**: 431 (1930).

ROBERTSON, A. C.: Preservation of Textile Fish Nets, *Ind. Eng. Chem.*, **23**: 1093 (1931).

——— and W. H. WRIGHT. Investigations upon the Deterioration of Nets in Lake Erie, *U.S. Dept. Comm.*, *Bur. Fisheries, Doc. 1083*, 1930

RUSCHMANN, G.: Faserstagebrösten mit Luftzufuhr, *Faserforschung*, **1**: 67 (1921).

———: Our Present Knowledge of the Retting Process of Flax, *Jour. Textile Inst.*, **15**: T61, T104 (1924).

SANBORN, J. R.: The China Blue-aurin Cellulose Medium for the Physiological Effect of Cellulose Destroyers, *Jour. Bact*, 14: 395 (1927).

SCHWARZ, E R.: "Textiles and the Microscope," McGraw-Hill Book Company, Inc., New York, 1934

SEARLE, G O : The Rotting of Textiles by Microorganisms   I   A Laboratory Test, *Jour. Textile Inst*, 20: T162 (1929)

SIEBER, W . Eine Prüfungsmethode für Wollfaserschädigungen, *Melliand Textilber*, 9: 326 (1928)

SKINKLE, J. H. "Elementary Textile Microscopy," Howes Publishing Co , New York, 1930

——— ; Physical and Chemical Textile Testing, a series of articles in *Am Dyestuff Rptr*, 25: P620, 649 (1936), etc

SMITH, A. L · A Contribution to the Bacteriology of Wool, *Am Dyestuff Rptr*, 21: 501 (1932).

SMITH, G . The Identification of Fungi Causing Mildew in Cotton Goods, the Genus Aspergillus, *Jour Textile Inst*, 19: T93 (1928).

SPENCER-SMITH, J L   An Electrical Method for Measuring the Moisture Content of Fabrics, *Jour Textile Inst*, 26: T336 (1935)

STEPHENSON, M . "Bacterial Metabolism," 2d ed , Longmans, Green & Company, New York, 1939

TAYLOR, H F , and A F. WELLS   Properties and Values of Certain Fish-net Preservatives, *U S Dept Comm*, Bur *Fisheries, Doc* 947, 1923, *Doc* 998, 1925

THAYSEN, A C., and H J BUNKER   Bacterial Decomposition of Cellulose, *Jour Roy Microscop Soc*, 43: 303 (1923)

——— and ———. "Studies of the Bacterial Decay of Textile Fibres   Variations in the Resistance of Cotton of Different Origins to Destruction by Microorganisms, *Biochem. Jour*, 18: 140 (1924)

——— and ——— : "The Microbiology of Cellulose, Hemicelluloses, Pectin and Gums," Oxford University Press, New York, 1927

THOM, C , H HUMFELD, and H P HOLMAN: Laboratory Tests for Mildew Resistance of Outdoor Cotton Fabrics, *Am Dyestuff Rptr*, Oct 22, 1934

TROTMAN, S R   Detection of Damage in Woolen Fabrics, *Textile Research*, 43: 70 (1925)

——— Detection and Control of Damage in Wool, *Ind Chemist*, 4: 118 (1928)

——— and R W SUTTON   The Influence of Micro-organisms in the Weathering of Wool and Cotton, *Jour Soc Chem Ind*, 43: T190 (1924)

——— and E R TROTMAN : "Textile Analysis," J B Lippincott Company, Philadelphia, 1932

UNNAN, S   Über die Zerstörung von Kunstseide und Mischgeweben durch Pilze, Bakterien und Insektenfrass, *Kunstseide*, 3. 90 (1936)

WAKSMAN, S A   "Principles of Soil Microbiology," The Williams & Wilkins Company, Baltimore, 1927

WATERS, R . Pink Rot of Wool, *Wool Record Textile World*, 41: 421 (1922).

WINOGRADSKY, S   Sur la décomposition de la cellulose dans le sol, *Compt rend*, 183: 691 (1926)

WORTHINGTON, C G , and C A JONES Flax Its Agricultural and Industrial Possibilities, *Am. Dyestuff Rptr* , 28: P63 (1939).

CHAPTER XXXVIII

THE MICROBIOLOGY OF WOOD

Wood may be injured or destroyed by several agencies, causing great losses annually. Fire is obviously the most serious enemy. Violent windstorms may be the direct cause of extensive destruction. Wear and weathering are inevitable. Animals, such as mice and rabbits, may do great damage by girdling fruit or other trees and causing their death. Biological agents alone cause the destruction of many millions of dollars worth of valuable wood each year. Included among the biological agents are the fungi—the principal biological causes of wood breakdown and chemical destruction; the insects—among which the termites are outstanding in importance; and molluscan and crustacean borers. Certain insects and borers are especially significant in the impairment or ruination of structures built of timber such as warehouses, wharves, trestles, and domestic buildings. From the standpoint of industrial microbiology, the changes produced in wood by the attacks of fungi are especially significant.

Structure and Composition of Wood.—It is not within the scope of this text to discuss in detail the structure of wood. In general, wood may be regarded as the mass of cells fully developed and modified during tree growth. It consists principally of lignin,[1] cellulose, and hemicelluloses with small quantities of starch, protein, and mineral salts.

Lignin is a constituent of the cell wall, to which it imparts strength and rigidity. Its exact chemical structure is unknown, but it is believed that the essential grouping of the molecule is coniferyl alcohol, coniferyl aldehyde, or a compound of closely related structure.[2]

Cellulose, $(C_6H_{10}O_5)_n$, a polysaccharide made up of a chain of beta-glucose residues joined at carbon atom 4 by glucosidal linkage,[2] when

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[1] SCHORGER, A. W., "The Chemistry of Cellulose and Wood," McGraw-Hill Book Company, Inc., New York, 1926, HAWLEY, L. F., and L. E. WISE, "The Chemistry of Wood," Reinhold Publishing Corporation, New York, 1926; NORMAN, A. C., "The Biochemistry of Cellulose, Polyuronides, Lignin, Etc ," Oxford University Press, New York, 1937, PHILLIPS, M , *Chem. Rev*, **14**: 103 (1934), HÄGGLUND, E., "Holzchemie," 2d ed., Akademische Verlagsgesellschaft m b H , Leipzig, 1939; FREUDENBERG, K., *Ann. Rev. Biochem*, **8**: 81 (1939).

[2] GORTNER, R A., "Outlines of Biochemistry," 2d ed , John Wiley & Sons, Inc , New York, 1938

hydrolyzed, gives rise first to cellobiose and then to glucose (see Chap. III).

Hemicelluloses include hexosans and pentosans, such as mannan, galactan, xylan, and araban, which yield mannose, galactose, xylose, and arabinose, respectively, when hydrolyzed by dilute acids. Levulose is also produced from certain hemicelluloses by hydrolytic processes. The hemicellulose content of hardwoods is greater than that of softwoods.

One analysis of spruce (*Picea excelsa*)[1] showed 30 per cent of lignin, 53 per cent of cellulose (free from pentosans), 15 per cent of hemicelluloses, and 2 per cent of fat, protein, resin, etc.

Ritter[2] of the Forest Products Laboratory of the U.S. Department of Agriculture has carried out research concerned with the structure of the cell wall, which has been dissected into layers, fibrils, fusiform bodies, and spherical units. Most of the lignin is present in the middle lamella, the rest occurring in other parts of the cell wall which is largely made up of cellulose and hemicelluloses

For a detailed discussion of the structure and chemistry of wood, the reader is referred to some of the publications listed at the end of the chapter.

THE FUNGI

The fungi here considered include certain forms of *Basidiomycetes* and *Ascomycetes* ("higher fungi") which actually bring about a disintegration of woody substances (decay, rot, and other types of breakdown); those which by pigment formation or by other means produce stains on timber, thus rendering the wood less valuable for some purposes; and "molds," including numerous forms of fungi imperfecti Wood-destroying fungi are, of course, both helpful and harmful in the economy of nature The breakdown of plant tissues, leaves, branches, and much fallen timber, either from natural or artificial causes, is advantageous, since the organic matter composing them is gradually decomposed and in large part returned to the soil as a result of the action of the enzymes manufactured by the fungi and the oxidations that follow If timber were not so broken down, it would accumulate in useless masses and interfere with forest growth On the other hand, wood-destroying fungi attack and seriously injure much valuable timber Logs may be destroyed before there is an opportunity for yarding them Sawed lumber, wooden ties, piles, sills, buildings, the supporting material of mine shafts, telephone and telegraph poles, fence posts, etc , are subject

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[1] SCHORGER, *op c t*
[2] RITTER, G J , *Paper Ind* , June, 1931

to the action of wood-destroying fungi, unless such wood is treated by chemical preservatives or otherwise to render it unsusceptible to attack.

The action of wood-destroying fungi is favored by the presence of moisture, warmth, and the absence of direct sunlight, as well as by the presence of oxygen and nitrogenous food materials that may be utilized by the fungi. On the other hand, there are certain volatile oils and water extractives contained in some heartwoods, as, for example, in cedar, that are toxic to fungi and therefore prevent or restrain decay for long periods.

Knowledge of the growth conditions of these organisms is of prime importance in procedures designed to prevent the action of wood-destroying fungi. For example, most fungi will not grow in wood containing less than 20 per cent moisture (on the basis of the oven-dried weight). On the other hand, the fungi will not grow when timber is submerged in water, or deep in the soil, because there is a deficiency of oxygen. Destruction of wood proceeds slowly or not at all in the cold weather of our Northern states, or in buildings maintained at low temperatures, but proceeds very rapidly in hot moist climates. Several types of chemical agents are very useful in preventing the action of fungi and in destroying them and are quite widely employed.

Some Important Wood-destroying Fungi.—Almost all the fungi that destroy wood are members of a few families of the Basidiomycetes.[1] Fungi belonging to the following genera are possessed of the ability to destroy wood. *Collybia, Lentinus, Pholiota, Pleurotus,* and *Schizophyllum* of the family *Agaricaceae; Echinodontium* and *Hydnum* of the family *Hydnaceae; Daedalea, Fistulina, Fomes, Gonoderma, Lenzites, Merulius, Polyporus, Poria,* and *Trametes* of the family *Polyporaceae;* and *Coniophora, Hymenochaete, Peniophora,* and *Stereum* of the family *Thelephoraceae.*

Enzymes of Wood-destroying Fungi.—The enzymes secreted by the wood-destroying fungi are responsible largely for the complex chemical changes that take place when the wood is attacked. Those fungi which completely disintegrate wood must elaborate enzymes that will attack the cell walls (cytase), the lignin (ligninase), the cellulose (cellulase), the hemicellulose (hemicellulase), and the various other substances present or formed as intermediates in the breakdown process.

The kinds and quantities of enzymes elaborated will depend on the species of fungus, the nature of the substrate, the pH, the temperature, and other factors. The following are some of the enzymes that have been reported as secreted by various wood-destroying fungi.[2] amidase, amylases, asparaginase, catalase, cellulase, cytase, emulsin, erepsin, ester-

[1] BOYCE, J. S., "Forest Pathology," McGraw-Hill Book Company, Inc., New York, 1938.

[2] BOSE, S. R., *Ergeb. Enzymforsch.*, **8:** 267 (1939).

ase, glucosidase, hemicellulase, hippuricase, inulase, invertase, laccase, lactase, ligninase, lipase, maltase, oxidase, pectinase, protease, raffinase, rennet, tannase, trypsin, tyrosinase, urease, and others.

Rots.—Two classes of rots are common in wood: the white rots, which may include pocket, stringy, flaky, or mottled rots; and brown rots, which include pocket, stringy, mottled, ring, or cubical rots

White rots are produced chiefly by fungi that attack the lignin principally, leaving white areas made up of cellulose compounds, and to a lesser degree by fungi that attack the cellulose and cause bleaching of the lignin.   White rots are especially addicted to Douglas firs but are found also in white pine, other conifers, hardwoods, mine timbers, etc   The red ring rot, whose causative agent is *Fomes pini* (*Trametes pini*), is a white pocket rot that attacks Douglas fir, ponderosa pine, and other woods It does not attack wood that is in use.   Red ring rot is the most serious cause of loss due to decay in this country.[1]   It attacks the heartwood particularly.   Hardwoods may be attacked by *Fomes applanatus* (the shelf fungus) when stored, or by *Fomes igniarius*.

Brown rots are commonly caused by cellulose-attacking fungi, although rots are not confined to these   The wood attacked by such fungi often may become so friable as to be pulverized by the fingers.   Brown rot fungi may attack the sound wood which is found between areas attacked by white rot fungi   Brown rots are frequently found in building timbers

Poria incrassata is the cause of brown cubical rot, a dry rot that destroys millions of dollars worth of coniferous timber in buildings in this country each year, especially in the states lying near the coasts of the Gulf and the Northwest   The fungus may transport moisture for several feet through its rhizomorphs   Thus it may attack and destroy wood which would otherwise remain dry   *Merulius lacrymans* produces a similar type of destruction of coniferous wood   This fungus, though common in Europe, is found infrequently in the United States.   It also possesses rhizomorphs that may transport water for some distance   Its growth is not favored by high temperatures

Brown rots of softwood are also caused by *Polyporus schweinitzii*, *Fomes pinicola*, *F. larices*, *Trametes serrales*, and other fungi.

Wood-staining Fungi.—The staining of wood may be caused in general by two different agencies chemical action, in which oxidizing enzymes are active in bringing about color changes in the sapwood, and fungi   The stains or discolorations produced by the fungi may be confined mainly to the surface of the woody materials, in which case they may be readily removed by planing or other treatment, or they may penetrate deeply into the wood rendering such removal out of the question

[1] Boyer, *op cit*

Stains may appear on wood products, on logs, or on dead or dying trees. The stains are usually confined to the sapwood, although occasionally the heartwood is affected. The presence of suitable food, moisture, and an optimum temperature favors the production by fungi of stains of various shades For example, blue stains, which are very common, are produced by *Cerastostomella, Alternaria,* and other molds; a grayish-black stain is caused by *Torula ligniperda;* a green stain by *Chlorosphenum aeruginosum;* a red stain by *Fusarium negundi;* and a yellow stain by *Penicillium divaricatum.*

In general, species of the following genera may produce discolorations that may be removed by planing, tbe use of steel brushes, or some other method: *Alternarium, Aspergillus, Cladosporium, Fusarium, Graphium, Mucor, Penicillium, Rhizopus, Torula* and others The stain or discoloration is usually produced as a result of the color formed in the mycelium of the mold, as a result of a soluble pigment, or as a result of a chemical reaction between some compound produced by the mold and the wood.

Staining may be prevented or controlled by drying the wood, by submerging it in water, by treating it chemically, or by other means. Drying of the wood is considered most effective; submergence is but a temporary measure. Occasionally wood may become stained before it can be dried. Resort may then be had to stain-preventing chemical dips Boyce[1] states that a dip composed of a 0.24 to 0.36 per cent solution of 6.3 per cent ethyl mercuric chloride, or a 0.5 to 1 per cent solution of equal parts of sodium tetrachlorophenoxide and sodium 2-chloro-orthophenylphenoxide, is effective in treating southern pines or hardwoods. Hardwood and red gum may be treated with a 5 per cent solution of commercial borax.

Hubert,[2] as the result of an extensive investigation involving the control of stain and decay in finished wood products (such as doors, window frames, and millwork in general) which are exposed to considerable moisture in service, has selected and recommended as being promising four chemical compounds out of the 25 chemicals and 18 proprietary wood preservatives he has studied. These chemical compounds included pentachlorophenol, *o*-phenylphenol, 2-chloro-o-phenylphenol (Permatol D), and tetrachlorophenol Hubert[3] has outlined the requirements for an effective preservative for exterior millwork, such as sash, door, and frame stock

For further details connected with the destruction and staining of wood by fungi, the reader is referred to the works of the Forest Products

[1] Boyce, *ibid.*

[2] Hubert, E. E, *Ind Eng Chem,* **30:** 1241 (1938).

[3] Hubert, E E, *Western Pine Assoc. Rev,* Tech Bull 6, Apr. 15, 1938.

Laboratory of the U.S. Department of Agriculture at Madison, Wis.; to the reports of the Bell Telephone System; to the texts listed at the end of this chapter—of which those by Boyce[1] and Hunt and Garratt[2] are of particular interest; and to the publications of the American Wood-preservers Association and the various schools of forestry.

Examination of Wood for the Presence of Fungi.—Wood may be examined for the presence of fungi by macroscopic, microscopic, cultural, and other methods. Discolorations may indicate decay, stains, or merely mold growth.

Cultural methods are used for the isolation of the agents producing decay and for the differentiation of various types. Media suitable for the desired purpose are used Bavendamm[3] used a 2 per cent malt agar containing 0 2 per cent tannic or gallic acid to differentiate between white-rot fungi and brown-rot fungi Colonies of the former fungi produced dark halos or oxidation rings on the special agar due to the oxidases that they secreted; while the latter fungi did not produce such diffusion zones. Davidson, Campbell, and Blaisdell,[4] as the result of extensive research, have shown that the generalization of Bavendamm was essentially correct They used 0.5 per cent concentrations of gallic or tannic acid in malt agar.

By the use of the microscope one may ascertain the presence of mold hyphae, boreholes (made by the perforation of the cell wall by hyphae), corrosion, and spiral cracks in the wood Hubert's stain is helpful in the foregoing connection.

Hubert's Stain for the Examination of Wood.[5]—Small pieces of wood, about 1 cm cubed, are boiled in water for 30 min and then soaked in a solution of equal parts of glycerol and ethanol until they become sufficiently soft to be cut readily with a razor blade

Thin sections are cut from the samples thus treated and immersed in a 2 per cent Bismarck brown solution (in 70 per cent ethanol) for 1 to 2 min , the time depending on the kind of wood, its density, the thickness of the sections and the degree of deterioration of the wood The excess of Bismarck brown is drained from the sections, which are then washed with distilled water The sections are next immersed in a solution of

[1] Boyce, *ibid*
[2] Hunt, G M, and G A Garratt, "Wood Preservation," McGraw-Hill Book Company, Inc , New York, 1938
[3] Bavendamm, W , *Zeit Pflanzenkrankh Pflanzenschutz* , 38: 257 (1928)
[4] Davidson, R W , W A Campbell, and D J Blaisdell, *Jour Agr Research*, 57: 683 (1938)
[5] Hubert, E E , *Phytopathology*, 12: 440 (1922), Thaysen, A C , and H J Bunker, "The Microbiology of Cellulose, Hemicelluloses, Pectins and Gums," Oxford University Press, New York, 1927

methyl violet (1 part of a saturated aqueous solution of methyl violet mixed with 3 parts of distilled water) for 2 to 5 min. Under certain circumstances it may be desirable to use the saturated solution of the dye, staining for 1 to 2 min. The sections are now washed with distilled water.

The stained sections are mounted in water on a glass slide and examined microscopically. If the violet color appears to be faint, the sections should be stained again with methyl violet. If the Bismarck brown is faint, the entire staining procedure should be repeated. Satisfactory sections are covered with a cover glass, to prevent the sections from curling, and dried slowly. (Egg albumen or gum arabic may be used to fix curled sections.) Permanent mounts may be made of the dried sections, using balsam or other material.

If a satisfactory stain, the mold hyphae become deep violet in color; the cell walls of the wood become yellow to brown; the wood tissues with exposed cellulose yield a mixed brown and violet color; and the contents of "medullary rays and the boarded pits of conifers" are dyed to a violet color usually.

BACTERIA

Bacteria and Wood Decay.—Bacteria exert a minor role in the decay of wood. Saprophytic types occasionally may increase the rate of decay by fungi. Under certain abnormal conditions cellulose-decomposing bacteria may bring about some breakdown of the wood. In general, it may be repeated that the higher fungi are mainly responsible for the destruction of wood.

There are several nonmicrobic biological agencies of wood destruction that are of great importance, and although they do not fall within the general scope of this work it may not be out of place to give a brief résumé of them here. For fuller information the reader is referred to works dealing specifically with these animals.

INSECTS

Termites.—Termites are insects that belong largely to the families *Termitidae, Kalotermitidae,* and *Rhinotermitidae.*[1] In the eastern part of the United States, *Reticulitermes flavipes* (Kollar) is a very common species.[2]

Termites occur where the climate is warm, generally in the tropical

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[1] KOFOID, C. A. *et al.*, "Termites and Termite Control," 2d ed., University of California Press, Berkeley, 1934.

[2] METCALF, C. L., and W. P. FLINT, "Destructive and Useful Insects," 2d ed., McGraw-Hill Book Company, Inc., New York, 1939, SNYDER, T. E., "Our Enemy the Termite," Comstock Publishing Company, Inc., Ithaca, N.Y., 1935.

and temperate regions. They cause great damage to wooden structures, the losses amounting to millions of dollars annually.

Although termites resemble ants in some respects and have been erroneously called "white ants," they are not ants. Their social organization, in some ways, is similar to that of ants. They live in colonies, a single colony sometimes containing thousands of individuals.

Workers (the termites largely responsible for building the colony, securing the food, and providing for the young) make up more than 75

Fig 120 —Work of the eastern subterranean termite in the sill of a house. Note the concentric arrangement of the passages due to leaving the harder summer wood in each annual ring. The knots are also not attacked. [*Courtesy of C H Blake, Tech Rev*, 41 (*No 3*) (1939)]

per cent of the termites. They are whitish in appearance and do not possess wings. They cannot produce young, for they are sterile.

Termites may be classified either as wood-dwelling or as subterranean termites.[1] The wood-dwelling termites include damp-wood termites and dry-wood termites. The former are found frequently in decaying, moist wood; while the latter are found in wood of relatively low moisture content.

The subterranean termites comprise a large part of the termites. The genera *Coptotermes*, *Reticulitermes*, and *Heterotermes*, of the family *Rhinotermitidae*, are known as "subterranean termites" and cause much of the destructive action of economic importance. These termites are widespread in distribution and are very devastating in their action. New colonies may start in the ground or on the surface of the ground, damp or partially decayed wood forming a good site. Moisture is required.

When subterranean termites attack the wood of a building, they may construct a covered passage between the colony and the site of their tun-

[1] Korow, *op cit*

neling operations.   The soil, wood, fecal matter, and other material they
use in building the passage form a plaster that is characteristic.

The initial attacks of termites are usually made on the outer walls
of a dwelling near the ground.   Such wood may appear to be sound even
though injured by termites.   By tapping on the wood or by pressing on
it, the damaged condition may be ascertained.

The termites cut off small pieces of wood with their jaws.[1]   The cel-
lulose present in the wood is broken down to sugars by the Protozoa that
are present in the stomachs of the termites.   The Protozoa possess
enzymes that have the ability to transform the cellulose, thus making
the wood available as food for the termites.
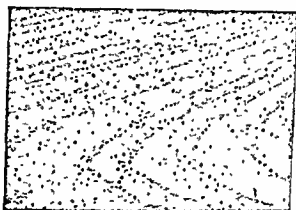
Fig. 121 —Surface of an oak board infested with powder-post beetles. The exit holes
are shown. They have a diameter of about $\frac{1}{16}$ in   [Courtesy of C. H. Blake, Tech Rev.
41 (No 3) (1939).]

Although many termites cause destruction of useful materials, some
are useful in returning dead wood to the soil

The prevention of destruction by termites may be largely achieved by
the use of proper construction methods.   Where termites are known to be
particularly destructive, materials that cannot be attacked should be
used.   The use of a shield of copper, or some other metal, between the
foundation and the structure (if the building is to be made of wood) is
advantageous.   When timbers may come into contact with the soil,
pressure impregnation with creosote is advocated.   Creosote oil is good
when discoloration, inflammability, and odor are not considered undesir-
able.   Timbers treated with zinc chloride are clean, odorless, and may
be painted.   Chromated zinc chloride is said to fix readily with wood and
to offer some resistance to fire.

For further information concerning termites and termite control, con-
sult some of the publications listed at the end of this chapter.

[1] Blake, C. H., New England Mus. Nat. Hist., Leaflet 3, 1937, Tech. Rev., 41
(No. 3), January, 1939

Powder-post Beetles.—The powder-post beetles (*Lyctidae*) are the most important of the insects producing the damage known as "powder-post defect." The adult females lay eggs in the wood. The latter develop into larvae, which live principally on starch. Tunnels of an irregular nature are excavated by the grubs. The fine powder resultant from their burrowings may fall out, producing characteristic heaps of wood sawdust. The injury caused by the powder-post beetles is confined to the sapwood of woods of broad-leaved trees such as ash, hickory, oak, and other woods.[1]
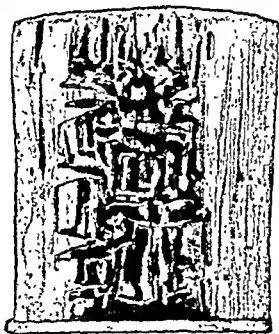
Fig 122 — Half section of part of a pole worked by carpenter ants. The true height of this specimen is 11 inches. [Courtesy of C H Blake, Tech Res, **21** (No 3) (1939).]

Carpenter Ants.—Carpenter ants are large black ants about 0 25 to 0 5 in in length. These insects dig tunnels and galleries in wood. The passages made by them are larger, in general, than those produced by termites and contain none of the plaster characteristic of the latter insect. The excavated wood is not used for food, the passages serving merely as shelters. Posts, structural timbers, and occasionally dwellings are attacked. The ants are very common in forests, where they may be found in stumps and in fallen or standing trees that are partially decayed

[1] *U S Dept Agr.*, *Bull.* 333 (1916), 1232 (1921), and *Farmers' Bull.* 1472 (Revised 1931)

THE MARINE BORERS

The marine borers include molluscan and crustacean borers. The molluscan borers, or shipworms, include the important genera *Teredo*, *Pholas*, *Martesia*, *Xylophaga*, *Lithodomus*, *Zirphaea*, and *Petricola*.[1]

Marine borers cause annually millions of dollar's worth of damage to wharves, docks, yachts, and other marine craft and structures. Of the marine borers, *Teredo* is by far the most destructive.
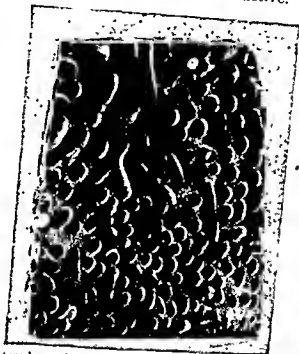
Fig. 123.—Work of teredos A block cut from damaged wood. Average diameter of the holes is about ¼ inch Note the white, limy lining. The passages do not intersect [Courtesy of C. H. Blake, Tech Rev., 41 (No 3) (1939)]

Teredo.—This borer is found in many parts of the world In this country it occurs on the Atlantic, Gulf, and Pacific coasts.

The female forms eggs that may be fertilized in its body or in the water, depending on the species. The eggs develop into larvae, which have bivalve shells and are free-swimming. It is during the larval period that the individuals are able to move about. After a few days the larvae swim to a structure or piece of wood and attach themselves. Entrance to the wood is made at right angles to the grain The tunnel thus made is of small diameter Later the teredo burrows with the grain, growing at the same time. Consequently the excavations become larger The teredo, once within the wood, never leaves it, the wood serving as a shelter and as part of its food supply.

The anterior end of the shipworm contains the valves, which are

[1] CLAPP, W. F., Civil Eng., 7: 105 (1937).

active in boring. The posterior end is supplied with two tubes; one of these takes in water (containing dissolved oxygen and food) and the other functions as an excurrent siphon to expel water, wood, wastes, and other substances.

The adult teredo sometimes reaches a length of 1 to 4 ft and a diameter of nearly 1 in Wood infested with these organisms will thus be weakened, the extent and rate of destruction depending on the numbers present; the species; the food available; the temperature, salinity, pH, and dissolved oxygen of the water; the presence or absence of pollution; and many other considerations.

Clapp[1] reports that *Teredo navalis* has been outnumbered by the larger and more destructive *T. megotara, T. dilatata, T. norvegiae,* and *T. Thompsoni* in New England harbors

Control of marine borers is, of course, very important. Research has done much to aid man in learning of the habits of the borers. Test boards have been suspended at various points in harbors and along the coast to determine the species and numbers present and the conditions favoring or inhibiting their presence. Certain test boards have been treated in various ways with chemical agents or by other means to discover methods of resisting the attack of the borers

Studies of associated organisms have yielded much information of value that may be used in predicting whether a certain location may at some future time be subject to the attacks of marine borers

Timbers and structures may be treated in various ways to resist attack. Wood impregnated with creosote is resistant. The protection of timbers by metal and masonry where they are exposed to water is of great value.

Boats may be protected by the application of special marine paints. According to Clapp, the timber keels, the yarboard planking, the shaft logs, and the centerboard wells are most susceptible to attack It is important to paint all exposed surfaces, for the borers may tunnel in on submerged portions where the boat is unprotected by paint

Martesia.—The individuals look much like small clams and may grow to a length of 2.5 in and a width of 1 in Much damage to marine structures is caused by this borer, principally on the Gulf of Mexico in this country

The young of this genus move about in the water without restriction. They bore into timbers when small, the entrances being usually ⅛ in. or less⁴ in diameter

Crustacean Borers. *Limnoria lignorum*—The destructive crustacean borers of importance include the isopods *Limnoria* and *Sphaeroma,* and

[1] Clapp, W. F , *Lab Bull* 5, Jan. 17, 1938
[2] Hunt and Garratt, *op cit.*

Hägglund, E.: "Holzchemie," 2d ed., *Akademische Verlagsgesellschaft m.b.H*, Leipzig, 1939.

Harlow, W. H., and E. S. Harrar: "Textbook of Dendrology," McGraw-Hill Book Company, Inc., New York, 1937.

Hawley, L. F., and L. E. Wise: "The Chemistry of Wood," Reinhold Publishing Corporation, New York, 1926.

Hill, C. L. and C. A. Kofoid: "Marine Borers and Their Relation to Marine Construction on the Pacific Coast," Report of the San Francisco Bay Marine Piling Committee, University of California Press, Berkeley, 1927.

Hubert, E. E.: Permatol, *Western Pine Assoc.*, *Tech. Bull.* 6, Revised April 15, 1938.

————: The Preservative Treatment of Millwork, *Ind. Eng. Chem.*, 30: 1222 (1938).

Hunt, G. M., and G. A. Garratt: "Wood Preservation," McGraw-Hill Book Company, Inc., New York, 1938

Koehler, A.: "Properties and Uses of Wood," McGraw-Hill Book Co., Inc., New York, 1924

———— and R. Thelen: "The Kiln Drying of Lumber," McGraw-Hill Book Company, Inc., New York, 1926.

Kofoid, C. A., et al.: "Termites and Termite Control," University of California Press, Berkeley, 1934.

Metcalf, C. L, and W P Flint: "Destructive and Useful Insects," 2d ed., McGraw-Hill Book Company, Inc , New York, 1939

Norman, A C.: "The Biochemistry of Cellulose, the Polyuronides, Lignin, Etc.," Oxford, Clarendon Press, New York, 1937.

Phillips, M.. The Chemistry of Lignin, *Chem. Rev*, 14: 103 (1934).

Record, S. J.: "Identification of the Timbers of Temperate North America, Including Anatomy and Certain Physical Properties of Wood," John Wiley & Sons, Inc , New York, 1934.

Ritter, G. J.: Newly Discovered Microscopic Structural Units of Wood Fibers, *Ind. Eng Chem.*, December, 1930

————: Structure of the Cell Wall of Wood Fibers, *The Paper Ind.*, June, 1934.

Schorger, A W.: "Chemistry of Cellulose and Wood," McGraw-Hill Book Company, Inc., New York, 1926

Snyder, T. E.: Preventing Damage by Lyctus Powder-post Beetles, *U S Dept. Agr*, *Farmers' Bull* 1477, March, 1926.

————: Defects in Timber Caused by Insects, *U.S. Dept. Agr.*, *Bull.* 1490, July, 1927.

————: Termites—Destroyers of Wood—and Man's Fight Against Them, *Proc. Am. Wood-Preservers Assoc.*, 25: 18–38 (1929)

————: "Our Enemy the Termite," Comstock Publishing Company, Ithaca, New York, 1935.

———— and J. Zetek: Damage by Termites in the Canal Zone and Panama and How to Prevent It, *U.S. Dept. Agr.*, *Bull.* 1232, 1924.

Thaysen, A. C., and H. J. Bunker: "The Microbiology of Cellulose, Hemicelluloses, Pectin and Gums," Oxford University Press, London, 1927.

Weiss, H F.: "The Preservation of Structural Timber," McGraw-Hill Book Company, Inc., New York, 1916.

Western Pine Association: *Tech Bulls*, Portland, Ore , 1935 *et seq.*

"What the Expert Knows about the Building Bogies, Termites and Rot," E I duPont de Nemours & Co , April, 1938.

"Wood Handbook; Basic Information on Wood as a Material of Construction with Data for Its Use in Design and Specifications," U.S. Dept. of Agriculture, Forest Products Laboratory, 1935.

APPENDIX A

DETERGENCY, DISINFECTION, AND STERILIZATION

The industrial microbiologist encounters other problems than those dealing with the cultivation and large-scale propagation of the organisms he is to employ as biochemical reagents for the transformation of organic materials. These problems involve the cleanliness and care of the equipment or apparatus he is to use, and the knowledge of how it can be made and kept free from invading microbes. Information on the general principles of detergency, disinfection, and sterilization will be found useful even though they have been worked out particularly in processes pertaining to public health and general sanitation.

Every bacteriologist recognizes the importance of employing sterile media and apparatus. Similarly, success in all technical fermentations depends on the application of some method or methods for inhibiting or destroying undesirable microorganisms, which are the ever-present enemies of the industrial microbiologist. The method used depends on the nature of the material to be sterilized and on the processes that may be affected. Where heat may be produced and applied cheaply without injury to the chemical character of the ingredients, steam is an excellent agent and the most reliable one to use for sterilizing mashes, fermentable solutions, etc., and for treating vats, pans, pipe lines, and other equipment. In certain industries, such as the acetone-butanol industry, where asepsis is so very important, steam, usually under pressure, is used in preference to all other agents. Other methods of sterilization, either physical or chemical, may be employed in individual cases, as in the sterilization of walls, floors, bench tops, etc. Before discussing these particular aspects of the subject further, it is well to define and explain the meanings of some of the terms used in connection with the destruction or inhibition of microbial life. Following this a brief enumeration of the types of agents available for this kind of work will be given.

Sterilization literally signifies the destruction of all living cells in a medium or environment. In commercial work the word is sometimes loosely employed to indicate a process of heating with the intent to kill germs. Heat is the most common sterilizing agent.

A *germicide* is anything, but especially a physical or chemical agent, that destroys germs. Patterson[1] states that "in practice it is assumed that a substance represented as a germicide, when used as directed, will kill all ordinary disease germs, but is not necessarily required to be capable of destroying bacterial spores." He further states that "in combatting diseases (such as anthrax or tetanus) caused by spore-forming bacteria, germicides or procedures specially effective against spores will be required." Obviously in technical fermentations the term "vegetative cells" should be substituted for "disease germs."

A "*bactericide* is anything that destroys bacteria." It does not necessarily destroy the spores of bacteria, however. Furthermore it is a more restricted term than germicide.

A *fungicide* is anything that destroys fungi.

[1] PATTERSON, A. M., *Am Jour. Pub. Health*, **22:** 465 (1932)

An *antiseptic* is a "substance that opposes sepsis, putrefaction or decay; one that prevents or arrests the growth or action of micro-organisms, either by inhibiting their activity or destroying them; used especially of agents applied to living tissues"[1]

Bacteriostasis is a term signifying a suspension of animation and reproduction by bacteria due to the influence of an agent known as a "bacteriostatic agent." It does not imply destruction of bacteria, although bacteriostatic agents, in high concentration or acting over a long period of time, may permanently destroy the ability of the bacteria to reproduce. The term "bacteriostasis" was introduced by Churchman in 1912. Some of the triphenylmethane dyes, such as crystal violet and brilliant green, and mercurials possess high bacteriostatic properties, especially in relation to certain Gram-positive bacteria.

Fungistasis, similarly, signifies a cessation of animation and reproduction by fungi.

A *disinfectant* is an "agent that frees from infection; usually, a chemical agent which destroys disease germs or other harmful micro-organisms (but not, ordinarily, bacterial spores), commonly used of substances applied to inanimate objects"[1]

Disinfection and sterilization will be discussed in this chapter primarily in relation to their industrial aspects.

Agents.—Agents for sterilization include physical and chemical agents. Physical agents include the use of high temperatures applied either as steam or dry heat for sterilization and pasteurization, the use of sunlight and ultraviolet light; the use of drying, pressure, high-frequency sound waves, or electrical currents; and filtration methods. Chemical agents include a very large list of substances from simple salts or soaps to special compounds, some of which are of complex structure. For the sake of simplicity, chemical agents may be classified on the basis of their chemical structure into acids, alkalies, phenols and phenol derivatives; salts and compounds of heavy metals, alcohols, halogens, dyes, and other compounds. In Table 173 several examples from the great number of the various classes of chemical agents that may be employed in disinfection with good results are given, although the agents mentioned are not necessarily better than many others unlisted.

TABLE 173—SOME CHEMICAL AGENTS USED AS ANTISEPTICS, GERMICIDES, OR DISINFECTANTS

Acids	Alcohols	Alkalies	Halogens	Heavy metals and compounds of them	Phenols and related compounds	Dyes	Miscellaneous
Acetic	Butyl (tertiary)	Sodium carbonate	Chloramines*				Alkyl sulfates
Benzoic	Ethyl	Sodium hydroxide	Azochloramid				
Boric		Trisodium phosphate	Chloramine-T				
Formic			Dichloramine-T				
Lactic			Chlorine	Metaphen	2-Chloro-o-phenyl-phenol	Crystal violet	
Salicylic			Hypochlorites	o-Hydroxyphenyl mercurie chloride	Cresols	Gentian Violet	
Sulphurous			Calcium hypochlorite	Phenylmercurie nitrate	Hexylresorcinol		
			Sodium hypochlorite	Silver (colloidal)	Pentachlorophenol		
			Fluorides	Silver compounds	Phenol		
			Iodine		o-Phenylphenol		
			Iodine compounds		Tetrachlorophenol		
					Thymols		

[1] *Ibid.*

The Phenol-coefficient Test.—This test is used in some form in nearly every civilized country in the world as the procedure for comparative rating of disinfectants It is a "standard" procedure used by the Food and Drug Administration,[1] which annually examines hundreds of samples of disinfectants, germicides, and bactericides Provided that a variety of conditions and a fairly large number of test organisms are used, this test may supply much valuable information. On the other hand it may require only a few tests to indicate that a substance has no particular value as a disinfectant.

Limitations of the Phenol-coefficient Test.—The phenol-coefficient test was designed to evaluate disinfectants that were closely related to phenol in chemical structure. This test is unsuited for the evaluation of substances quite unlike phenol and for substances insoluble in water. It gives no information concerning the ability of the chemical agent to penetrate organic matter, nor its toxicity to tissues.

Definition of Phenol Coefficient.—The phenol coefficient is a term that expresses the germicidal action of a chemical agent toward a test organism at a given temperature in terms of the action of phenol under identical conditions Thus a phenol coefficient of 5 0 means that an agent is five times as effective as phenol under a given set of conditions, which should be specified Should a phenol coefficient be stated on the label of a container without the name of the test organism or the temperature of the test, then it is understood that the test was carried out with *Eberthella typhosa* (Hopkins strain) at a medication temperature of 20°C.

E. typhosa (Hopkins strain), which may be secured from the Food and Drug Administration at Washington, D C , is the standard test organism used in testing disinfectants This organism is cultivated under a standard set of conditions, as outlined in *Circular 198, Food and Drug Administration, U.S Department of Agriculture*, and should demonstrate a fairly constant resistance to phenol at 20° and 37°C. Should its resistance vary appreciably, a new subculture should be obtained for test purposes

Calculation of the Phenol Coefficient.—To calculate the phenol coefficient, the denominator of the fraction expressing the highest dilution of the chemical agent that destroys the test organism in 10 but not 5 min is divided by the denominator of the fraction expressing the highest dilution of phenol that destroys the test organism in 10 but not 5 min. under the same set of conditions

Let us suppose that a test was carried out according to Food and Drug Administration methods, using *Staphylococcus aureus*, Government 209, a special strain that may be used in testing germicides and antiseptics, as the test organism at a medication temperature of 20°C , and let us suppose that the data shown in the table on page 850 were obtained

The phenol coefficient, calculated from these data, is $\frac{125}{50} = 2.5$

Procedure in the Phenol-coefficient Test.—The Food and Drug Administration test is made in the following manner Dilutions of phenol and of the disinfectant, or germicide, are prepared in a series of sterilized medication tubes (Pyrex tubes) with a diameter of 25 mm and a height of 150 mm) Just 5 cc of the chemical agents or of dilutions of them are left in each tube The tubes are placed in a rack in a water bath adjusted to the desired temperature and permitted to stand for at least 5 min. before the test is carried out, in order to bring the temperature of the contents of the tubes to that of the bath The bath should be provided with an efficient stirrer and an accurate thermostat capable of maintaining the temperature within 0 1°C. or less, of that desired

The test culture, which should be 24 hr old (22 to 26 hr) and which should be

subcultured successively at daily intervals for at least 5 days before use, in order to activate it, is shaken vigorously to break up the small clumps of bacteria that may be present and is placed in the water bath at least 15 min. before the test is carried out. Sufficient of the test culture is drawn into a single sterile pipette to inoculate every tube of the series. To each 5 cc. portion of the chemical solutions in the medication tubes is added 0.5 cc. of a broth culture of the test organism. At intervals of 5, 10, and 15 min., a 4-mm. loopful (No. 23 B. & S. gauge platinum wire) of material is withdrawn from each tube and planted in a corresponding tube of Reddish broth, or of the medium that is especially adapted for the growth of the organism being studied. The subculture tubes are incubated at the temperature most favorable for the growth of the test organism. Preliminary observations may be made after 24 hr., but final observations should not be made until the end of 48 hr. Under certain conditions, it may be advisable to incubate the subculture tubes for longer periods of time, especially if a substance suspected of possessing high bacteriostatic properties is being examined.

Chemical agent	Dilution	Time intervals, minutes		
		5	10	15
Phenol	1/60	+	0	0
Phenol	1/70	+	+	+
Disinfectant	1/120	0	0	0
Disinfectant	1/130	0	0	0
Disinfectant	1/140	0	0	0
Disinfectant	1/150	+	0	0
Disinfectant	1/160	+	+	+
Disinfectant	1/170	+	+	+
Disinfectant	1/180	+	+	+
Disinfectant	1/190	+	+	+

NOTE.—A + sign signifies that *Staphylococcus aureus* was not destroyed by the chemical agent at the time interval indicated, while a 0 sign indicates that it was destroyed.

In the case of mercurials or other agents possessing high bacteriostatic properties, it is essential to make secondary subcultures from the subculture tubes immediately after the test has been completed. This may be accomplished by transferring one to four 4-mm. loopfuls of material from each subculture tube to corresponding tubes of sterile culture medium. Secondary subculture tubes should be incubated along with the primary subculture tubes. Growth in the secondary tubes but not in the primary tubes is a reliable indication of the bacteriostatic nature of the chemical agent being examined. Other indications of bacteriostasis are the appearance of growth in primary subculture tubes later than 48 hr., or the appearance of "skips" or of irregularities in the data secured from the 48-hr. observation.

Occasionally a chemical substance may be added to the subculture medium, which neutralizes any bacteriostatic effects. For example, hydrogen sulphide may be added in the case of mercuric chloride, or sodium thiosulphate, in the case of hypochlorites

The phenol coefficient is calculated from the data of the experiment, provided

they are adequate. It is always desirable to duplicate results. Estimated values should be substantiated by further tests.

It is to be repeated that substances quite unlike phenol should not be examined by this method but by others that are more appropriate.

For other details concerning this test the student is referred to *Circular* 198, *Food and Drug Administration, U.S. Department of Agriculture* (December, 1931).

Since the publication of the U.S. Food and Drug Administration Methods of Testing Disinfectants and Antiseptics, several methods for evaluating germicides and antiseptics have been reported. Although these are not now to be regarded as standard, they offer, in many instances, substantial improvements in methods of evaluation. They may be regarded as other types of measuring sticks.

Requirements of a Disinfectant.—The requirements of a disinfectant will depend on the purposes for which it is to be used. In general, a disinfectant should possess high germicidal activity, should be effective in the presence of organic matter and at the pH and temperature used, should be stable, should be water soluble (for many purposes), should impart no undesirable color or odor, should possess no undue toxicity for animals, and should be capable of being produced at a reasonable cost. Obviously it is not an easy matter to produce a chemical agent that is ideal in all ways.

A chemical agent would have little value if it did not possess the ability to destroy various types of bacteria, molds, and/or yeasts, pathogenic and nonpathogenic, under the conditions of use. Since most substances of this nature are ineffective or merely antiseptic in very dilute form, the disinfectant must not be used in too low a concentration if it is to be effective. A useful method for calculating a satisfactory dilution is to multiply the so-called "phenol coefficient" (a figure obtained under special conditions at 20°C., using *Eberthella typhosa* (Hopkins strain) as the test organism) by 20. The reciprocal of this figure is the dilution that may be used. For example, a disinfectant with an *E. typhosa* phenol coefficient of 5 might be employed in a dilution equal to the reciprocal of 20×5, or $\frac{1}{100}$, that is, 1 part of disinfectant in 99 parts of water. This is the concentration that corresponds to a $\frac{1}{20}$, or 5 per cent, phenol solution.

Almost all disinfectants are reduced in chemical efficacy by the presence of organic matter. This is true of hypochlorites, mercurials, and other compounds. Phenol and cresols are not so much affected by organic matter as certain other disinfectants.

The reaction at which a disinfectant is used is at times very important. Hypochlorites are very much more effective at a slightly acid reaction. Certain other compounds are more efficacious when the pH is relatively high. The flavines, for example, acriflavine, are more effective when the reaction is alkaline.

Temperature is a very important factor in disinfection. It is a well-known fact that chemical reactions usually proceed more rapidly as the temperature is elevated. Disinfectants, as a rule, are more effective at 37°C. than at 20°C. or at lower temperatures. There are apparent exceptions to this rule, however.

It is important to have information concerning the solubility of the disinfectant in water and other solvents. For most practical applications of disinfection, water-soluble types of chemical compounds are essential, as, for example, in treating a water supply or in sterilizing the walls of tanks, pipes, etc. Under certain conditions it may be desirable to impregnate substances, such as fish nets, tents, or tarpaulins with a substance that will not be washed out when the fabric comes into contact with water, as it must sooner or later.

Stability is an important requirement for a disinfectant. Most chemicals are not used at once after their manufacture. They may remain for months as unsold stock

Even after purchase by the ultimate consumer, the agent may not be used immediately. It is, therefore, important that the disinfectant should be stable or that the approximate rate of its decomposition be known. Cognizance is taken of these facts in the distribution of liquid hypochlorites. For this type of disinfectant, it is important that the date of manufacture, the quantity of available chlorine at that date, expressed in percentage, and the approximate rate of decomposition when stored in a cool, dark place at a temperature not higher than 20°C. be known.

Ways and means for aiding in the stabilization of relatively unstable compounds are known in many cases. Hypochlorites may be partially stabilized by adjusting the pH to extreme alkalinity through the use of sodium and/or calcium hydrates, sodium carbonate, or other suitable alkaline substances. Obviously the pH of these compounds must be adjusted to a much lower value for effective germicidal action. This may be done at the time of use. Peroxides may be stabilized by the use of small amounts of acetphenetidide or of acetanilide or by other means. For example, Perhexogen is a hydrogen peroxide in a solvent of tertiary butanol with a trace of metaphosphoric acid as a preservative.[1]

Under some circumstances it may make no special difference whether a disinfectant imparts a color or not, but usually one that does not add color is desirable. Creosotes are excellent disinfectants for the treatment of wood, but they cannot be used on surfaces that are to be painted, or the inner surfaces of vats or tanks.

The question of odor is an important one also. Phenols and cresols cannot be used in food establishments near foods on account of the readiness with which many types of foods absorb odors. One part of phenol in several million parts of water may be readily detected by the average individual, and its odor may be imparted to foods even when the dilution is great. Compounds must not be used to treat interior woodwork if they evolve undesirable odors.

Disinfectants intended for general plant purposes where there is likely to be contact with the hands or skin must not possess too great toxicity. In the disinfection of stables, it is essential to use a disinfectant which will destroy pathogens but which will not injure livestock. Certainly disinfectants used in the treatment of equipment that is used in connection with manufacture of food or beverages *must not possess high toxicity*. Chlorine compounds, chlorine, and hypochlorites are especially valuable for the latter purposes. Scales[2] has pointed out the possibilities of using alkyl aryl sulphonate, either alone or with a chlorine solution. Spores were readily destroyed by low concentrations of the compound.

The question of toxicity to tissues is, of course, of paramount importance in the case of germicides and antiseptics.

Chlorine Solutions.—Chlorine ... ... several types. aqueous solutions of chlorine, hypochlorite ... ... Chlorine is much used in the treatment of ... ... water used for cleaning fish, and for many ... ... dium hypochlorite (NaOCl), which has been made alkaline with sodium hydroxide, calcium hydroxide, sodium carbonate, or some other alkaline salt; and calcium hypochlorite, ... hypo-

than 7. Zonite, Bacili Kil, Eusol, and Perchloron are hypochlorite ...

The chloramine compounds include chloramine-T (p-toluene sodium sulphon-

[1] COMBES, F. C., *N.Y. State Jour. Med*., Nov. 15, 1937.

[2] SCALES, F. M., paper presented to Laboratory Section of International Association of Milk Dealers, Oct. 17, 1938.

chloramide), dichloramine-T (p-toluene sulphon-dichloramide), and Azochloramid (N,N'-dichloroazo-dicarbonamidine). Chloramine-T is water soluble and liberates hypochlorous acid in aqueous solution. Dichloramine-T is insoluble in water, it is mixed with a chlorinated oil. Azochloramid liberates chlorine very slowly and is said to produce good results in surgical antisepsis.

A commercial standard for "liquid hypochlorite disinfectant, deodorant and germicide" (CS 68-38) became effective on June 10, 1938 [1] Herein it is stated that the chlorine content of a hypochlorite solution shall appear on the label and shall not be less than 2.5 per cent When stored in the original container in a dark place at a maximum temperature of 20°C. (68°F.), the rate of deterioration of the compound shall not be greater than 10 per cent of the original content of available chlorine in 6 months.

Considerable research concerning chlorine solutions has been carried out Significant papers have been presented by Johns, Charlton and Levine, Costigan, and others As is the case with many disinfectants, temperature, pH, and concentration are very important factors in connection with the efficiency of germicidal action. Hypochlorites and chloramine-T solutions are strongly affected by pH changes, being very much more effective germicidally in slightly acid solutions than in alkaline solutions. Chloramine solutions appear to be less affected by pH changes [2]

Charlton and Levine are of the opinion that the undissociated hypochlorous acid (HOCl) is the most important germicidal agent of hypochlorites Undissociated hypochlorous acid is apparently not the significant factor in the case of simple chloramines or chloramine-T, where it is believed that the positively charged chlorine atom is mainly responsible for the disinfecting action

In general, the germicidal efficiency of chlorine and hypochlorites is considerably reduced by the presence of organic matter. Accordingly, it is essential to clean a surface thoroughly before applying the chlorine solution.

Quaternary Ammonium Compounds.—These are surface-active agents, which are widely used as sanitizing agents and germicides They are active in relatively high dilutions and are relatively nontoxic, noncorrosive, free of color, tasteless and odorless in the concentrations usually employed There are soluble in water They have a wide range of compatibilities, but are incompatible with soap, certain anionic compounds, and some detergents.

Information concerning the quaternary ammonium compounds has been reviewed by Rahn and Van Eseltine (1947), Lawrence (1947), Varley (1947), Rucker, Brooks, Metcalf, and Van Eseltine (1947), Dunn (1949), and others The use of these compounds in the brewing industry has been described by Lehn and Vignolo (1946)

The quaternary ammonium compound is one built around the nitrogen atom that contains five valence bonds Four of these bonds are attached to adjacent carbon atoms of organic radicals and one is attached to an inorganic or organic radical Both noncyclic and cyclic quaternary ammonium compounds are manufactured for use as sanitizers and germicides The formulas of different types of these compounds are presented in Table 174

Detergents.—A detergent is a substance that cleanses

The more common detergents include caustic soda (NaOH), soda ash or sodium carbonate (Na₂CO₃), trisodium phosphate (Na₃PO₄), sodium metasilicate, and sodium

[1] U S Dept. Comm., Nat Bur. Standards, Com Standard, CS68-38, 1938
[2] Charlton, D , and M. Levine, Iowa State Coll Agr Mech. Arts, Eng. Expt. Sta , Bull 132, 1937.

hexametaphosphate [(NaPO₃)₆ or Na₂(Na₄P₄O₁₃)]. Various mixtures of the fore-going are employed.

Caustic soda forms the base of several cleaning solutions. The concentration of sodium hydroxide used will depend upon the type of work being done. The American Bottlers of Carbonated Beverages state that a minimum of 3 per cent alkali solution should be used in treating unclean bottles, of which not less than 60 per cent (1 8 per cent) must be caustic soda The bottles must be exposed to this solution for not less than 5 min. at a temperature of not less than 130°C. An "equivalent cleansing and sterilizing process" may be substituted for the foregoing. The usual concentration of sodium hydroxide used may vary from 0.5 to 4 per cent. Many bacterial spores and other resistant bacteria are destroyed under these conditions. For example. Arnold and Levine have reported that *Staphylococcus aureus* was destroyed in 10 min.¹ at 98 6°F, by a 1.5 per cent concentration of caustic soda.

Trisodium phosphate is much used as a cleaning compound. It is less efficient as a germicide than caustic soda. Trisodium phosphate, soda ash, sodium meta-silicate, and sodium hexametaphosphate are used in combination with caustic soda to produce more efficient cleansing. Caustic soda produces good deflocculation and emulsification but rather poor wetting and rinsing results. Trisodium phosphate and sodium metasilicate when mixed with caustic soda improve the efficiency of the combination, for these compounds are good rinsing agents, in addition to their other qualifications. Sodium hexametaphosphate aids in the removal of bacteria from glassware. Surface-active agents may increase the efficiency of cleaners.

In any washing operation, the efficiency of the detergents will depend on the thoroughness with which the process is carried out mechanically; the nature and amount of the soil, the kinds and quantities of the detergents used; the ability of the detergents to emulsify and saponify the fatty components of the soil; the solution, wetting, and rinsing abilities of the detergents, the temperature of the cleansing water; the germicidal action, and other factors.

During recent years considerable information concerning the detergent value of various compounds has been published. A few references to such literature will be found immediately following

References on Detergency, Disinfection, and Sterilization

American Medical Ass.

Arnold, C R, and M i . ance with Bottle   ages, Washington, 1938.

Baker, C. L.: Detergent Value of Sodium Metasilicate, *Ind. Eng Chem.*, 23: 1025 (1931)

Bronfenbrenner, J., A D. Hershey, and J. A. Doubly: Evaluation of Germicides by a Manometric Method, *Proc. Soc. Exptl. Biol Med.*, 38: 210 (1938); *Jour Bact.*, 37: 583–597 (1939).

———, ———, ———: Verification of the Results Secured by the Manometric Method of Evaluation of Germicides, *Jour. Bact.*, 36: 265 (1938).

Bryan, A. H.: Modified Reddish Cup Technique for Evaluating the Germicidal Activity of "Liquid Antiseptics," *Jour. Am. Pharm. Assoc.*, 27: 654 (1938)

Carswell, T. S., and J. A. Doubly: Germicidal Action of Benzylphenols, *Ind Eng. Chem*, 28: 1276 (1936).

¹ Arnold, C R, and M. Levine, American Bottlers of Carbonated Beverages, Washington, D.C., 1938.

TABLE 174.—THE CHEMICAL STRUCTURES OF SOME QUATERNARY AMMONIUM COMPOUNDS[1]

Name of compound	Chemical structure of compound	Trade name
Alkyldimethylbenzylammonium chloride	$\left[C_nH_{2n+1}\text{—}\overset{+}{N}(CH_3)_2\text{—}CH_2\text{—phenyl} \right]$ Cl⁻	No-Roma, Onyx BTC, Roccal, Roclina, Rodalon, Zephiran, Zephirol
Cetyldimethylbenzylammonium chloride	$CH_3(CH_2)_{15}\text{—}\overset{+}{N}(CH_3)_2\text{—}CH_2\text{—phenyl}$ Cl⁻	Triton K-12
Diisobutylphenoxyethoxyethyldimethylbenzylammonium chloride	$(CH_3)_2CHCH_2\ C(CH_3)_2\text{—phenyl—}O\text{—}(CH_2)_2\text{—}O\text{—}(CH_2)_2\text{—}\overset{+}{N}(CH_3)_2\text{—}CH_2\text{—phenyl}$ Cl⁻	Amersep, Hyamine 1622, Phemerol, Polymrin D
Cetyltrimethylammonium bromide	$CH_3(CH_2)_{15}\text{—}\overset{+}{N}(CH_3)_3$ Br⁻	CTAB, Cetavlon, CETAB
Cetylpyridinium chloride	$CH_3(CH_2)_{15}\text{—}\overset{+}{N}\text{(pyridinium)}$ Cl⁻	Ceepryn
N-(higheralkyl)esters of rohamino(formyl-methyl) pyridinium chloride	$CH_3(CH_2)_n\text{—}O\text{—}\overset{O}{C}\text{—}(CH_2)_2\text{—}\overset{H\ O}{N}\text{—}C\ CH_2\text{—}\overset{+}{N}\text{(pyridinium)}$ Cl⁻	Emulsept

[1] For an extensive review on the subject, refer to the chapter on the Quaternary Ammonium Compounds in "Advances in Food Research," Vol. II, Academic Press Inc., New York, 1949.

CARTER, J. D.: The Value of Silicate of Soda as a Detergent, II, *Ind. Eng. Chem.*, **23**: 1389 (1931).

CHARLTON, D., and M. LEVINE: Germicidal Properties of Chlorine Compounds, *Iowa State Coll. Agr. Mech. Arts, Eng. Expt. Sta*, Bull. 132, 1937.

DUNN, C. G.: Quaternary Ammonium Compounds in the Food Industries, chapter in "Advances in Food Research," Vol. II, Academic Press, Inc., New York, 1949.

ETCHELLS, J. L., and F. W. FABIAN: Technique for Skin Irritation Tests, *Jour. Ind Hyg.*, **17**: 298 (1935).

GARROD, L. P.: The Testing of Disinfectants in the Presence of Organic Matter, *Jour. Hyg.*, **35**: 219 (1935).

JOHNS, C. K.: The Evaluation of the Germicidal Potency of Chlorine Compounds, *Sci. Agr.*, **14**: 585 (1934).

HEINEMAN, P. G.: Critical Review of Methods of the Evaluation of Antiseptics, *Arch. Path*, **26**: 320 (1938).

HUCKER, G. J., R. F. BROOKS, D. METCALF, and W. VAN ESELTINE: The Activity of Certain Cationic Germicides, *Food Technol.*, **1**: 321–344 (1947).

HUMFELD, H.: The Sterilization of Wool and Its Effect on the Physical and Chemical Properties of a Wool Fabric, *U.S. Dept. Agr., Tech. Bull.* 588, September, 1937.

HUNT, G. A.: The Use of Cutaneous *Staphylococcus* Lesions in Mice for the Evaluation of the Germicidal Activity of Disinfectants, *Jour. Infectious Diseases*, **60**: 232 (1937).

HUNT, G. M., and G. A. GARRATT: "Wood Preservation," McGraw-Hill Book Company, Inc, New York, 1938

KLARMANN, E.: Scientific Advances in 1937 in Disinfectants and Antiseptics, *Soap*, January, 1938

LAWRENCE, C. A.: Quaternary Ammonium Germicides More Expensive than Most, They Compete on an Efficiency Basis, *Chem. Inds.*, **60**: 44–47 (1947).

LEHN, G. J., and R. L. VIGNOLO: Applications of Quaternary Ammonium Compounds in the Brewing Industry, *Brewers Digest*, **21**: 41–44 (1946)

MALLMANN, W. L.: A Critical Study of Various Types of Detergents and Disinfectants for Use in Dishwashing, *Am. Jour. Pub. Health*, **27**: 464 (1937).

MARSHALL, M. S., and A. K. HRENOFF: Bacteriostasis, *Jour. Infectious Diseases*, **61**: 42 (1937).

McCULLOCH, E. C.: "Disinfection and Sterilization," 2d ed, Lea & Febiger, Philadelphia, 1945

NYE, R. N.: The Relative in Vitro Activity of Certain Antiseptics in Aqueous Solution, *Jour. Am. Med. Assoc.*, **108**: 280 (1937).

"Pharmacopoeia of the United States of America," 13th revision, Mack Printing Co., 1947.

RAHN, O., and W. P. VAN ESELTINE: Quaternary Ammonium Compounds, *Ann Rev. Microbiol.*, **1**: 173–192 (1947).

REDDISH, G. F.: Recent Developments in Methods of Testing Germicides, *Ind Eng Chem.*, **10**: 425 (1938).

——— and E. BURLINGAME: A Laboratory View of Athlete's Foot. *Druggists Circular*, June, 1938.

——— and ———: A Method for the Bacteriologic Testing of Chemical Solutions Used for the "Cold Sterilization" of Surgical Instruments, *Jour. Bact*, **35**: 265 (1938).

SALLE, A. J., W. A. McOMIE, I. L. SHECHMEISTER, and D. C. FOORD: An Improved Method for the Evaluation of Germicidal Substances, *Proc. Soc. Exptl. Biol Med.*, **37**: 694 (1938).

Schwartz, C., and B. H. Gilmore. Sodium Metaphosphate in Mechanical Dish-washing, *Ind Eng. Chem.*, **26**: 998 (1934).

Stericker, W.: The Value of Silicate of Soda as a Detergent—1. *Ind. Eng. Chem*, **15**: 244 (1923).

U.S. Dept. of Agriculture: Some Common Disinfectants, *Farmers' Bull*. 926, 1931.

U.S. Dept. of Commerce: Pine Oil Disinfectant, *Comm. Standard* CS69-38, 1938.

U.S. Dept. of Commerce: Coal Tar Disinfectant, *Comm Standard* CS70-38, 1938.

U.S. Dept. of Commerce: Cresylic Disinfectants, *Comm. Standard* CS71-38, 1938.

Vail, J. G.: The Role of Silica in Soluble Silicate Cleansers, *Ind. Eng. Chem.*, **28**: 294 (1936).

Varley, J C.: Practical Aspects of the Quaternaries, *Soap Sanit. Chemicals*, **23** (No. 12): 130–133, 165 (1947).

APPENDIX B

THE TREATMENT AND DISPOSAL OF
INDUSTRIAL MICROBIOLGICAL WASTES

Fermentation methods are of great service in the treatment of industrial wastes or of domestic sewage when the amount and character of the organic matter is such as to serve as a substrate for bacterial or other microbic action. Such fermentations are sometimes based on an effort to utilize as fully as possible the fermentable material with the production of some gaseous or other, product such as methane, which might be utilized as a source of heat energy in the industrial operation of the plant. In other instances the aim is to degrade the organic matter by fermentations so that it is no longer putrescible, or so that the amount of putrescible matter eventually discharged into sewers, streams, or other bodies of water will be reduced to a minimum and thus produce little or no effect on the fish or other natural inhabitants of the waters receiving such partially purified wastes.

The disposal of wastes from industrial plants presents special problems in some localities and in particular types of industries on account of the relatively large content of organic matter and the high biochemical oxygen demand (B.O.D.) of these wastes. Indeed the extra load placed on sewage treatment plants by some of these industrial plants may be the equivalent of the load from the inhabitants of a good-sized city.

Wastes from plants utilizing raw materials rich in carbohydrates, proteins, or fats offer special problems in purification by microbic methods. Thus the wastes from the manufacture of corn sugar or starch; from sugar mills; from milk, butter, and cheese plants; or from canneries and slaughtering and meat-packing establishments may cause serious nuisances and high pollution of the waters into which the sewage is discharged unless there is a high degree of breakdown of organic matter previous to such discharge

Water is used for many purposes in an industrial plant,[1] the most important of which is in connection with some aspect of processing of materials. Process water, which of course varies considerably in amounts, according to the industry, contains a relatively large amount of organic matter This may be dissolved, colloidal, or in fine particles that settle on standing. For example, process liquors may contain from 1 to 7 per cent of total solids. The inorganic matter and settleable solids account for less than 50 per cent of the total solids, while much of the organic matter is in true solution and is not removed by the ordinary processes of coagulation. Likewise, ultrafiltration does not remove the matter in true solution, whereas it may reduce the B.O.D. of domestic sewage to zero. Thus industrial sewage differs from domestic sewage in several respects.

Liquid wastes, whether treated or not, are generally disposed of ultimately by the method of dilution, in which they are discharged into streams of fresh water or into large bodies of water, such as the Great Lakes, or tidal waters such as bays. In a small percentage of cases land treatment may be used.

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[1] BUSWELL, A. M., *Water Works and Sewerage*, April, 1935.

Provided that they have been stabilized or sufficiently diluted, after screening to remove large floating objects, or filtered to take out suspended matter, no nuisances or destructive action will generally arise as the result of the disposal of wastes from fermentation plants. But should there be a lack of dissolved oxygen in the streams as a result of improper treatment of the wastes prior to the disposal of them, nuisances may arise and the water may be rendered incapable of supporting fish and other desirable forms of life.

In general, two methods, one aerobic, the other anaerobic, may be used to stabilize sewage, although various modifications and combinations of the two may be used. In the aerobic method, in which the effluent is of low color and B.O.D., the organic compounds are decomposed with the formation of carbon dioxide, water, and a small residue consisting largely of colloidal and suspended matter. Among the recommended aerobic methods are trickling filters and activated-sludge treatments. In the anaerobic method, methane, carbon dioxide, and a humus-like solid are the main products formed, according to Buswell.[1] The ratio of methane to carbon dioxide varies generally from 1:1 to 3:1, depending on the nature of the wastes and the conditions of the fermentation. A wide variety of organic waste materials, with the exception of mineral oils, may be fermented to produce these gases. The methane evolved may be used as a fuel, while the carbon dioxide might be recovered if in sufficient quantity and used in the manufacture of dry ice or for some other purpose. When conditions permit, an anaerobic method in which the methane formed is used for fuel may be the most economical one for the treatment of trade wastes from large plants. Although there is a 75 to 90 per cent reduction[2] in the B.O.D., this, as well as the color, is still relatively high after anaerobic treatment. Therefore, such effluents are improved by aerobic treatment.

The control of acidity is important in the treatment of industrial wastes, for example, of beer slop from distilleries and of brewery steep water. Wastes that are highly acid cannot be treated directly by aerobic methods, even when the load is small. However, such wastes when neutralized or diluted from 1:10 to 1:100 with water or domestic sewage may be suitably treated. The limit of acidity in the anaerobic treatment when undisturbed continuous fermentation is desired is approximately 2,000 p.p.m., calculated as acetic acid[3]

The wastes are recovered in the greater number of the large industrial plants, but if the liquid wastes are too dilute, recovery of products is impracticable, and they may then be stabilized by treatment at the plant or by the city treatment plants.

Some Present Methods of Treating and Disposing of Wastes.—Wastes may be disposed of or utilized in several different ways. They may be disposed of by dilution without any preliminary treatment, other than screening. This is done in the case of some plants situated in localities where the sewage is conveyed out to sea or dumped into other bodies of water. Wastes may be anaerobically decomposed and the methane produced used for fuel. The wastes from certain fermentation plants and food manufacturing plants may be concentrated and used as a food for livestock. Certain wastes may be concentrated and, on account of their potassium content, used as a constituent of fertilizers. In rum and whisky plants, from 15 to 35 per cent of the liquid residue from distillation (the distillation slops) is generally used in addition to a fresh supply of water in preparing new mashes. Wastes from small plants, which do not operate continuously, may be disposed of by broad irrigation or lagooning. Other methods for treating wastes are being investigated.

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[1] Buswell, A. M., *Ind. Eng. Chem.*, **31:** 1349–1351 (1939)

[2] *Ibid.*

Wastes from Industrial-alcohol Plants.—In an industrial-alcohol plant that uses molasses as the raw material, the slops may have a total solid content of 5 per cent,[1] organic solids amounting to 4 per cent, and a 5-day B.O.D. of about 22,000 p p m—a high pollution load. Such wastes may be concentrated in multiple-effect evaporators and then incinerated. The ash, which contains approximately 33 per cent potassium oxide, is sold to fertilizer manufacturers. When the price for potash is high, the recovery of this slop is worth while. Otherwise *recovery adds to the cost of producing alcohol.*

Some concentrated slop is dried and marketed as chicken feed. Such material is laxative in nature and hygroscopic.

Such wastes may be digested anaerobically and stabilized aerobically.

Wastes from Yeast Plants—The wastes from a yeast plant ordinarily contain 1 to 3 per cent of total solids[2] and have a 5-day B.O.D. of 7,000 to 14,000 p p m. This waste is too weak to justify the expense of recovery for stock feed, but, on the other hand, the load is sufficiently high to cause serious pollution of streams Such wastes may be digested *anaerobically and then stabilized aerobically by treatment on trickling filters.*

Wastes from Breweries.—The liquid wastes from a brewery include those from the brewer's grain, from the recovery of yeast, and from the wash water of the various departments of the brewery. The waste from brewer's grain may contain 3 per cent solids and have a 5-day B.O.D. of 10,000 to 25,000 p p m It has been estimated that the combined wastes from a brewery, per 31-gal barrel of beer, are equivalent to those of a population load of 15 to 25 persons[3] Such wastes, however, are too dilute to warrant recovering them as stock feed. They may be disposed of either separately or together with domestic sewage through the usual standard methods of treatment.

Wastes from Distilleries.—The slops from the stills of a distillery may contain from 4.75 to 6 per cent of total solids[3] and 2.5 to 3 per cent of soluble solids and may have a 5-day B.O.D. of 15,000 to 20,000 p.p.m. The slop and wash water from each bushel of grain ground usually amounts to 45 to 55 gal. The solids remaining after the distillation, known as "distiller's grains," are sold as stock feed.

In one large distillery, the liquid wastes are screened, the suspended solids are removed by centrifuges, and the liquor is then evaporated in multiple-effect evaporators. The material thus recovered is sold as a feed.

In large plants in general, the wastes are screened and the screenings dried. The slop may be evaporated, dried, and incorporated with the dried screenings The combined recovered product amounts to about 18 lb. per bushel of the originally ground grain [3]

In small distilleries, on the other hand, the slop may be screened, the screenings pressed and dried, and the weak slop disposed of along with domestic sewage. Usually 8 to 10 pounds[3] of dried material are recovered from each bushel of grain ground.

The wastes from a distillery, which contain 3 to 4 per cent of total solids and 0 2 per cent of organic acids, and are still hot, may be fermented by thermophilic bacteria with the production of a mixture of methane and carbon dioxide, at low cost. From 58 to 72 per cent of the organic matter is gasified in 2 to 6 days. The residual sludge is inoffensive and stable, while the liquid wastes may be safely disposed of to the sewers.

Wastes from Acetone-butanol Plants—The slop from the acetone-*butanol industry*

[1] Boruff, C S , *Ind. Eng. Chem.*, **31**: 1335–1337 (1939)
[2] *Ibid*

in which molasses is fermented may have a 5-day B.O.D. of 7,000 to 11,000 p p m ¹ It is thus a heavy waste. This waste may be digested anaerobically and stabilized by aerobic treatment. Some slop is concentrated, dried by the drum method, and distributed to stock-feed mixers as a source of vitamin G, for such concentrates contain 70 to 100 micrograms of this vitamin per gram

Wastes from Dairy Plants.—Very large quantities of dairy wastes are discarded each year to the sewer. The wastes include those from pasteurizing and bottling plants, from creameries, and from cheese factories. The total solid content of these wastes may vary from a fraction to about 4 per cent. Chemical precipitation of milk wastes is of little value in reducing the pollution load, since the lactose and other soluble solids that are not precipitated are readily attacked by bacteria and other microorganisms.

A very dilute milk waste will cause no trouble when disposed of along with other sewage. However, dairy wastes may frequently be the cause of considerable nuisance—the failure of sewage-treatment processes to operate successfully, the destruction of the normal life of a stream, etc

Dairy wastes may be treated by one of the standard methods for treating domestic sewage or they may be fermented anaerobically according to the method developed by Boruff and Buswell,² wherein methane is produced as an important end product and is utilized for fuel purposes

After certain preliminary treatment, which may involve the removal of settleable solids and grease, dairy wastes with a total solid content of no greater than 0 05 to 1 per cent³ may be efficiently treated by one of the standard aerobic filter methods The wastes may be passed through trickling filters with capacities for 600,000 to 2,000,000 gal of liquid wastes per acre per day, through lath filters with capacities for 250,000 to 2,250,000 gal per acre per day, or through sand filters, which may have capacities for 50,000 gal per acre per day.¹

Milk wastes may be disposed of by broad irrigation, but not infrequently disagreeable odors may arise from lagoons unless the wastes are prechlorinated.

In an anaerobic method recommended by Buswell and his associates,² 05 per cent, or greater, of the pollution load may be removed, while 8 3 to 12 4 cu ft of gas may be produced from each pound of dried solids added to the fermentation tanks The filtered effluent from these tanks may be further treated, if desired, by one of the standard filter methods. The cost of this treatment, in which methane and carbon dioxide are the main gases produced, with small quantities of hydrogen and nitrogen, has been estimated by Buswell and his associates to be considerably less than that for present-day standard methods

The anaerobic fermentation process developed by Boruff and Buswell may be carried out in a single tank or in two tanks connected in series. Before dairy wastes are introduced, the tank is filled about one-third full with well-digested sewage sludge and asbestos fibers and the remaining two-thirds with over-flow liquor (which has been permitted to settle) from an anaerobic sewage tank. The asbestos fibers serve as a rest or support for the bacteria after the sludge has been consumed. The sludge and overflow liquor supply the initial medium and the starter for the subsequent

¹ *Ibid*

² Boruff, C S, and A M Buswell, *Ind. Eng Chem*, **24**: 33 (1932).

¹ Buswell, A M, C S Boruff, and C K Wiseman, *Ind Eng Chem*, **24**: 1423 (1932)

fermentations. The temperature is maintained at 27 to 29°C. The dairy wastes are fed into the tank slowly during its operation.

Organic matter is believed to be broken down to organic acids, such as propionic and acetic acids, by the anaerobic bacteria, which then convert these acids to methane and carbon dioxide, principally. If, during the fermentation, wastes are introduced too rapidly, organic acids may accumulate at the expense of gas production. An undesirable flora may thus develop. Accordingly, during the first part of its operation, especial care must be exercised to favor the production of methane and carbon dioxide and a desirable bacterial flora in the tank. Should the contents of a tank become sour, the rate at which milk wastes are fed should be reduced or even stopped for a while. When two tanks are operated in series, liquor from the normal tank may be run into the contents of the tank that has become too acid. This practice usually restores a normal fermentation.

From 1.6 to 2.4 volumes of gas may be produced from each tank volume per day, with no "noticeable" quantity of sludge.

Wastes from Canneries.—Information concerning methods of treating cannery wastes may be obtained by studying *Bulletin 28-L* of the Research Laboratory of the National Canners Association.[1]

Additional Information.—The reader desiring detailed information concerning the biological, chemical, and engineering aspects of this subject may obtain it by studying some of the excellent texts and articles cited in the following list of references. Buswell and Hatfield's publication on "Anaerobic Fermentations" will be found of particular value. This publication contains a large number of references to the literature, including patent references, which are important in this as well as in other fields.

References on Industrial Waste Disposal and Allied Subjects

BABBITT, H. E.: "Sewerage and Sewage Treatment," 4th ed., John Wiley & Sons, Inc., New York, 1932.

BORUFF, C. S.: Waste Problems in the Fermentation Industry, *Ind. Eng. Chem.*, **31**: 1335–1337 (1939).

BUSWELL, A. M.: "The Chemistry of Water and Sewage Treatment," Reinhold Publishing Corporation, New York, 1928.

———: The Treatment of "Beer Slop" and Similar Wastes, *Water Works and Sewerage*, April, 1935.

———: Biological Processes for Treating Waste, *Ind. Eng. Chem.*, **31**: 1349–1351 (1939).

——— and W. D. HATFIELD: Anaerobic Fermentations, *State Water Survey*, *Ill. Bull.* 32, 1939.

DRINKER, P.: Atmosphere Pollution, *Ind. Eng. Chem.*, **31**: 1316–1320 (1939).

DUNHAM, G. C.: Military Preventive Medicine, 3d ed., Army Med. Bull. 23, Carlisle Barracks, Pa. (1939).

EHLERS, V. M., and E. W. STEEL: "Municipal and Rural Sanitation," 3d ed., Mc-Graw-Hill Book Company, Inc., New York, 1943.

Federation of Sewage Works Associations, "Modern Sewage Disposal," New York, 1939.

FULLER, G. W., and J. R. McCLINTOCK: "Solving Sewage Problems," McGraw-Hill Book Company, Inc., New York, 1926.

[1] WARRICK, L. F., F. J. McKEE, H. E. WIRTH, and N. H. SANBORN, December, 1939.

GAINEY, P. L.: "Microbiology of Water and Sewage," Burgess Publishing Co., Minneapolis, 1939.

HOOVER, C. R.: Treatment of Liquid Wastes from the Textile Industry, *Ind. Eng. Chem.*, **31**: 1352–1358 (1939).

HOWE, H. E., and F. J. VAN ANTWERPEN: Utilization of Industrial Wastes, *Ind. Eng. Chem.*, **31**: 1323–1330 (1939).

METCALF, L., and H. P. EDDY: "American Sewerage Practice," Vol. I, "Design of Sewers" (1914), Vol. II, "Construction of Sewers" (1915), Vol. III, "Disposal of Sewage" (1915), McGraw-Hill Book Company, Inc., New York.

——— and ———: "Sewerage and Sewage Disposal," McGraw-Hill Book Company, Inc., New York, 1930.

NELSON, G. H., R. P. STRAKA, and M. LEVINE: Effect of Temperature of Digestion, Chemical Composition, and Size of Particles on Production of Fuel Gas from Farm Wastes, *Jour. Agr. Research*, **58**: 273–287 (1939).

PARKER, M. E.: "Food Plant Sanitation," McGraw-Hill Book Co., Inc., New York, 1948.

PRESCOTT, S. C., and M. P. HORWOOD: "Sedgwick's Principles of Sanitary Science and Public Health," The Macmillan Company, New York, 1935.

ROSENAU, M. J.: "Preventive Medicine and Hygiene," 6th ed., D. Appleton-Century Company, Inc., New York, 1935.

SKINNER, H. J.: Waste Problems in the Pulp and Paper Industry, *Ind. Eng. Chem.*, **31**: 1331–1335 (1939).

TOBEY, J. A.: Legal Aspects of the Industrial Wastes Problem, *Ind. Eng. Chem.*, **31**: 1320–1322 (1939).

TOWNSEND, D. W.: Relation of Industrial Wastes to Sewerage Problems, *Eng. Contract Record*, **52** (No. 42): 36, 38 (1939).

WARRICK, L. F., F. J. MCKEE, H. E. WIRTH, and N. H. SANBORN: Methods of Treating Cannery Waste, *Nat. Canners Assoc. Bull.* 28-L, Washington, December, (1939).

WESTON, R. S.: Water Pollution, *Ind. Eng. Chem.*, **31**: 1311–1315 (1939).

Some Patents

CARP-THOMAS, G. H.: Bacteriological Digester for Conversion of Organic Waste, U.S. Patent 2,178,818, Nov. 7, 1939.

WILKIE, H. F., and C. S. BORUFF: Process for the Dehydration of Moisture-containing Compounds, U.S. Patent 2,165,950, July 11, 1939.

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